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REMARKS/ARGUMENTS

Claims 13-40 are pending. Claims 33 and 34 have been allowed. Claims 13-16, 22, 29 and 30 have been amended to refer to alpha-glycated peptides or oxidases reactive with alpha-glycated peptides. Claim19 has been further limited to alpha-glycated dipeptides, which are described in the specification on page 4, line 6. Other editorial revisions have been made for clarity. Accordingly, the Applicants do not believe that any new matter has been added.

The Applicants thank Examiner Leary for the courteous and helpful interview of May 11, 2004. The differences between the method of the present invention, which is now directed to a method for assaying an α-glycated peptide using (2) an oxidase which reacts with α-glycated peptides and prior art methods which do not suggest a method for assaying an alpha-glycated peptide were discussed. The Applicants were encouraged to further elaborate on the differences between the oxidases of the invention and the FLOD-S oxidase disclosed by Yonehara, col. 4. These comments are presented below. Accordingly, favorable consideration is now requested.

Rejection—35 U.S.C. 102(b)

Claims 1, 6, 8 and 9 were rejected under 35 U.S.C. 102(b) as being anticipated by, or in the alternatively, under 35 U.S.C. 103(a) as being unpatentable over Yonehara et al., U.S. Patent No. 5,985,591.

Initially, the Applicants reiterate their argument that the cited prior art does not disclose or suggest an oxidase which reacts with glycated peptides. Conventional amino acid oxidases which deglycosylate glycated amino acids were not disclosed as having any activity on glycated peptides. This is further highlighted by a subsequently published scientific article: Hirokawa et al. (2003, attached) which discloses "Frustosyl amino acid oxidase

(FAOX) or amadoriase, catalyzes the oxidative deglycation of glycated amino acids to produce the corresponding amino acids" (page 104, bottom of col. 1), but that "the FAOX's characterized previously could not be applied to the enzymatic determination of HbA1c, since they did not have activity toward fructosyl peptides" (page 104, bottom of col. 2). Thus, while several proteases are disclosed by Hirokawa as liberating fructosyl peptides from HbA1c, these could not be assayed using conventional fructosyl amino acid oxidases. Based on the disclosure of Hirokawa, the Applicants submit that if fructosyl amino acid oxidases which react with fructosyl peptides were not known in 2003, then they also were not known in 2000, when the present application was filed.

Similarly, the conventional frucotsyl amino acid oxidases (FAODs) disclosed by Yonehara et al., col. 3, lines 49-65, e.g., oxidases (1)-(6) and "FLOD-S" (col. 4, lines 54-56) are only disclosed as reacting with glycated amino acids. However, the present claims are directed to a method which uses an oxidase that reacts with an α-glycated peptide. Yonehara does not disclose with sufficient specificity nor suggest a method using an oxidase which reacts with an α-glycated peptide.

To anticipate an invention, the prior art must allow one to immediately envisage the invention or disclose the invention with sufficient specificity. Moreover, to establish anticipation regarding an alleged inherent characteristic, the Office must provide evidence that makes clear that the missing descriptive matter is *necessarily* present, Continental Can Co. USA v. Monsanto Co., 20 USPQ2d 1746, 1749 (Fed. Cir. 1991); MPEP 2131.02 (III). Assuming *arguendo*, that some of the FAODs disclosed by Yonehara et al. inherently have some degree of activity on glycated peptides, one would not have immediately envisaged an assay method for glycated peptides involving these oxidases, because there is no disclosure or suggestion in Yonehara for such a method. Thus, Yonehara could not have disclosed the claimed invention with sufficient specificity to rise to anticipation, nor suggest the invention

for purposes of obviousness. Moreover, the Office has presented no evidence that the oxidases of Yonehara *necessarily* have any activity on glycated peptides. Accordingly, the Applicants respectfully request that this rejection now be withdrawn for the reasons set forth above.

To further distinguish the claimed methods from Yonehara, the claims are now directed to methods involving α-glycated peptides using an oxidase which reacts with α-glycated peptides. In an α-glycated peptide the α-amino group of the amino acid residue at the amino terminal of the peptide has been glycated. Yonehara does not disclose with sufficient specificity, nor suggest, a method for assaying for the presence of an α-glycated peptide or a method which uses an oxidase which reacts with α-glycated peptides.

The oxidase of the present invention reacts with an α-glycated peptide. As discussed above, at the time of filling of the present application, oxidases known to react with glycated peptides or polypeptides were unknown. For example, the conventional fructosyl amino acid oxidase ("FAOD") derived from the genus *Corynebacterium* does not react therewith. This conventional FAOD is included in oxidases as exemplified in Yonehara et al. (column 3, lines 49-65). Yonehara et al. also describe that "FLOD-S," described in Publication of Japanese Patent Application (Tokkai Hei) No. 7-289253, was used as FAOD in the Examples thereof, and that FLOD-S specifically effects fructosyl lysine and fructosyl-N'-2-lysine ("FNZ") (see column 4, lines 54-62). FLOD-S is further described by Sakai, Y. et al., Biosci. Biotech. Biochem., 59(3), 487-491 (1995). Sakai was written by some of the inventors of Japanese Patent Application (Tokkai Hei) No. 7-289253 cited in col. 4, line 56, of Yonehara. A FLOD described in Sakai, Y. et al. and the FLOD-S are both derived from *Fusarium oxysporum* S-1F4. Therefore, the FLOD-S is identical to the FLOD described in Sakai, Y. et al. In Sakai, Y. et al., the following matters are described: FLOD (which is identical to the FLOD-S) showed low activity against fructosyl valine; FLOD had activity against N^{ε}-

fructosyl-N^{α}-Z-lysine(ε-FL) and N^{α}-fructosyl-N^{ε}-Z-lysine(α-FL), but the Km (Michaelis constant) was lower for ε-FL than for α-FL. Sakai concludes on page 491, first column that FLOD seems to recognize glycation in the ε-amino group (see Sakai, Y. et al., page 489, right column, "Substrate specificity" paragraph, and page 491, left column, lines 2-7). Therefore, it appears that the glycated peptide described in Yonehara et al. is the glycated peptide in which the ε-amino group of lysine residue has been glycated. Thus, Yonehara et al. does not disclose or suggest selection of oxidases which react with an α-glycated peptide.

Moreover, the methods exemplified by Yonehara et al. use glycated peptides glycosylated at *internal* lysine residues. Serum is mainly composed of albumin and globulin. Generally, it is known that albumin and globulin are glycosylated at lysine residues inside the proteins; see e.g., Iberg. N. and Fluckiger, R., J., Biol. Chem., 261, 13542-13545 (1986, attached). Accordingly, the Applicants respectfully request that this rejection be withdrawn.

Allowable Subject Matter

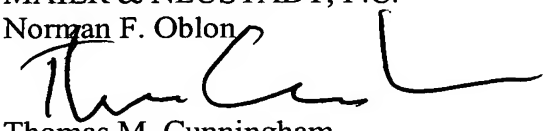
The Applicants thank Examiner Leary for indicating that Claims 33 and 34 are allowed and that the subject matter of Claims 18, 21, 23-28, 30, 36 and 37 is otherwise in condition for allowance.

CONCLUSION

In view of the above amendments and remarks, the Applicants respectfully submit that this application is now in condition for allowance. Early notification to that effect is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.
Norman F. Oblon



Thomas M. Cunningham
Registration No. 45,394

Customer Number
22850

Tel: (703) 413-3000
Fax: (703) 413 -2220
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Molecular cloning and expression of novel fructosyl peptide oxidases and their application for the measurement of glycated protein

Kozo Hirokawa,* Keiko Gomi, and Naoki Kajiyama

Research and Development Division, Kikkoman Corporation, 399 Noda, Noda-city, Chiba Pref. 278-0037, Japan

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Abstract

Fructosyl peptide oxidases, enzymes that are active against a model compound of glycated hemoglobin, N^{α}-fructosyl valyl-histidine, were characterized. To identify the primary structure of fructosyl peptide oxidases, we have prepared cDNA libraries from *Eupenicillium terrenum* ATCC18547 and *Coniochaeta* sp. NISL9330. The coding regions, both fungal fructosyl peptide oxidases consisting of 1314-bp, were obtained with degenerated primers based on the amino acid sequences and specific primers by 3′ and 5′ RACE (rapid amplification of cDNA ends). By their sequence similarities and substrate specificities, fructosyl peptide oxidases and their homologs could be categorized into two groups: (A) enzymes that preferably oxidize α-glycated molecules and (B) enzymes that preferably oxidize ε-glycated molecules. We showed that recombinant fructosyl peptide oxidases could be used to detect protease-treated fructosyl-hexapeptide, a glycated peptide that is released from HbA_{1C} by endoproteinase Glu-C, suggesting these enzymes could be useful for the enzymatic measurement of HbA_{1C}.

Keywords: Hemoglobin A_{1C}; Fructosyl peptide oxidase; Fructosyl amino acid oxidase; Amadoriase; Fructosyl-hexapeptide; Enzymatic measurement of HbA_{1C}; *Eupenicillium terrenum*; *Coniochaeta* sp.

Glycation is the non-enzymatic reaction by which Amadori compounds are produced from reducing sugars, such as glucose, and an amine. In vivo, the levels of glycated blood proteins reflect the level of glucose, since blood proteins, such as hemoglobin and albumin, are readily glycated by glucose at their amino groups [1,2]. For clinical diagnosis, the development of a selective and sensitive method for the measurement of glycated hemoglobin (HbA_{1C}) and albumin is of considerable importance. HbA_{1C} is glycated at the N-terminal valine residue of the β-subunit [3], while the glycated sites of albumin are the internal lysine residues of the protein. Therefore, N^{α}-fructosyl valine (Fru-Val) and N^{ε}-fructosyl lysine (εFru-Lys) were applied as model compounds of HbA_{1C} and glycated albumin and an extensive search was carried out to find an enzyme that shows specificity for such fructosyl amino acids.

Fructosyl amino acid oxidase (FAOX), or amadoriase, catalyzes the oxidative deglycation of glycated amino acids to produce the corresponding amino acids, glucosone, and hydrogen peroxide [1]. FAOXs have been isolated from *Corynebacterium* sp., *Agrobacterium* sp., *Aspergillus* sp., *Penicillium* sp., and *Fusarium* sp. [4–7]. We have shown that the bacterial FAOXs characterized to date are distinct from eukaryotic FAOXs, based on sequence comparisons and substrate specificities [8]. Bacterial FAOXs had sequence similarity with an opine-catabolizing enzyme in *Agrobacterium* and were active against Fru-Val [8,9]. Substrate specificities of eukaryotic FAOXs have also been determined and it was found that εFru-Lys and fructosyl propylamine are good substrates for FAOXs from *Fusarium* sp., *Aspergillus* sp., and *Pichia* sp. [10–12].

To develop enzymatic determination of HbA_{1C}, we have found several proteases that could efficiently liberate fructosyl peptides from HbA_{1C} (our unpublished result). However, the FAOXs characterized previously could not be applied to the enzymatic determination of HbA_{1C}, since they did not have activity toward fructosyl peptides. Therefore, the primary aim of our study was to find a novel fructosyl peptide oxidase (FPOX) that is

* Corresponding author. Fax: +81-4-7123-5550.
E-mail address: khirokawa@mail.kikkoman.co.jp (K. Hirokawa).

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active against fructosyl peptides and that could be applied to the measurement of HbA1c. Previously, we screened a fungal culture collection for FPOX using Nε-fructosyl valyl-histidine (Fru-ValHis) as a substrate. We found that the cell extracts of eight genera, *Achaetomiella*, *Acheatomium*, *Chaetomium*, *Coniochaeta*, *Eupenicillium*, *Gelasinospora*, *Microascus*, and *Thielavia*, showed FPOX activity [13]. In the present study, we purified and characterized two FPOXs; one from *Eupenicillium terrenum* ATCC18547 and one from *Coniochaeta* sp. NISL9330. Both enzymes have high activity toward Fru-Val and Fru-ValHis, however they differed in specificity toward εFru-Lys. cDNA coding for FPOX was cloned from the cDNA library of *E. terrenum* and *Coniochaeta* sp. Primary structures were compared to those of known FAOXs in order to elucidate the relationship between the catalytic properties and the protein structure.

Materials and methods

Materials

N-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline, sodium salt (TOOS) was purchased from Dojindo Laboratories, Japan, and horseradish peroxidase was from Kikkoman, Japan. Fru-ValHis and fructosyl amino acids were prepared as described previously [4].

Methods

Assay of FPOX activity. FPOX activity was measured spectrophotometrically at 37 °C with the peroxidase-coupled reaction system, as described previously [8]. The standard reaction mixtures contained 0.1 M potassium phosphate, pH 8.0, 0.5 mM TOOS, 900 U/l. of peroxidase, 0.45 mM of 4-aminoantipyrine, and 20 mM Fru-ValHis in a final volume of 3 ml. One unit of enzyme activity was defined as the amount of enzyme that produced 0.5 μmol of quinoneimine dye per minute at 37 °C.

Preparation of cell extracts and purification of FPOX. E. terrenum ATCC 18547 was grown aerobically at 25 °C for 96 h in 6 L of medium (0.1 g of yeast extract, 0.1 g of malt extract, 0.1 g of KH_2PO_4, and 0.05 g of $MgSO_4$ in 100 ml, pH 7.3). Washed mycelia were suspended in TEG buffer (10 mM Tris–HCl buffer, pH 8.0, containing 1.0 mM EDTA and 1.0% glycerol) and disrupted by a French press. The homogenate was centrifuged at 9000*g* for 30 min to remove intact cells and cell debris. Ammonium sulfate was added to the above supernatant to 40% saturation and then the precipitate formed was pelleted by centrifugation at 15,000*g* for 10 min. The supernatant to which ammonium sulfate was added to 60% saturation was centrifuged at 15,000*g* for 10 min. The resultant precipitate was dissolved in TEG buffer and dialyzed. The dialyzed enzyme solution was applied to an Ultrogel AcA34 (IBF Biotechnics, France) equilibrated with TEG buffer. Gel filtration chromatography was carried out with the same buffer. The active fractions were collected and dialyzed, and then applied to a Q Sepharose FF column (Amersham Biosciences) equilibrated with TEG buffer. The absorbed protein was eluted with a linear NaCl gradient (0–0.5 M). The active fractions were collected and dialyzed by ultrafiltration, and then applied to a Poros R2/M column (PerSeptive Biosystems, Japan) equilibrated with TEG buffer containing 2.0 M ammonium sulfate. The absorbed protein was eluted with a linear gradient of ammonium sulfate (2.0–0 M) in TEG buffer. The specific activity of the purified preparation was 3.6 U/mg. Purification of FPOX from *Coniochaeta* sp. NISL 9330 was carried out as described previously [13].

Protein methods. Protein was measured with a Bio-Rad protein assay kit, using bovine serum albumin as a standard. The molecular mass of each purified enzyme was determined by gel filtration chromatography on a G3000SWXL column (7.8 mmØ × 30 cm; Tosoh, Japan) equilibrated with 20 mM potassium phosphate, pH 8.0, containing 5% glycerol and 0.15 M NaCl. Glutamate dehydrogenase, lactate dehydrogenase, enolase, myokinase, and cytochrome *c* were used as molecular standards. The molecular masses of the subunit were determined by SDS–PAGE. The purified FPOXs from two fungi were partially digested with trypsin or endoproteinase Lys-C (Roche Diagnostics, Japan), processed with ABI 173A MicroBlotter system (Applied Biosystems, Japan), and then blotted onto a PVDF membrane. The amino acid sequence of the peptide in each spot was determined by Edman's method with a protein sequencer (Applied Biosystems model 476A).

RNA Isolation and reverse transcriptase-mediated PCR. Total RNA was isolated from *E. terrenum* and *Coniochaeta* sp. using Isogen (Wako, Japan), essentially according to the manufacturer's instructions. cDNA libraries were synthesized from the total RNA with oligo(dT) primers and Avian-Myeloblastosis virus reverse transcriptase (Takara, Japan). Degenerate PCR oligonucleotides were designed using the peptide sequences generated from the Edman degradation reactions. By using the cDNA library of *Eupenicillium* along with the 5′-degenerated primer (primer 1, Table 3) and 3′-degenerated primer (primer 2), a fragment of *Eupenicillium* FPOX cDNA was obtained by degenerated PCR. Similarly, a fragment of *Coniochaeta* FPOX cDNA was obtained using the cDNA library of *Coniochaeta* with the 5′-degenerated primer (primer 3) and 3′-degenerated primer (primer 4) by degenerated PCR.

3′ and 5′ RACE. Specific primers were synthesized for 3′ and 5′ RACE on the basis of the partial nucleotide sequence of *Eupenicillium* and *Coniochaeta* cDNAs. For 3′ RACE of *Eupenicillium* FPOX cDNA, 3′ RACE primer (primer 5) and oligo(dT)-adapter primer (Takara, Japan) were used. For 5′ RACE of *Eupenicillium* FPOX cDNA, the 5′-Full RACE core set (Takara, Japan) was used with the anchor primer (primer 6). 1st primer pairs (primers 7, 8), and 2nd primer pairs (primers 9, 10). Similarly, 3′ RACE primer (primer 11) and oligo(dT)-adapter primer were used for 3′ RACE of *Coniochaeta* FPOX cDNA. The anchor primer (primer 12), 1st primer pairs (primers 13, 14), and 2nd primer pairs (primers 15, 16) were used for 5′ RACE of *Coniochaeta* FPOX cDNA.

PCR amplification. The *Eupenicillium* and *Coniochaeta* cDNAs were added to the reaction mixture as a template for PCR (2.5 mM $MgCl_2$, 20 pmol of primers, PCR buffer, and 2.5 U of *Taq* polymerase). Amplification consisted of denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 90 s.

Cloning of PCR products. PCR products were analyzed by electrophoresis on a 1% agarose gel (Takara, Japan). They were cloned into a linearized pT7Blue T-vector (Novagen, UK) and subsequently introduced into *Escherichia coli* JM109 cells. Recombinant plasmids were selected by blue white selection and sequenced using the CEQ 2000XL DNA Analysis System and CEQ Dye Terminator Cycle Sequencing kit (Beckman Coulter, USA).

Subcloning for FPOX genes. For the amplification of the coding region of the FPOX-E transcript, an upstream primer (primer 17) and a downstream primer (primer 18), corresponding to the amino acid sequences of the N- and C-terminal regions of FPOX-E, were used. PCR was carried out in 50 μl of a reaction mixture containing 50 pmol of each primer, 120 mM Tris–HCl, pH 8.0, 1.0 mM $MgCl_2$, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, and 0.2 mM dNTP. Thirty cycles (98 °C for 15 s, 65 °C for 2 s, and 74 °C for 30 s) of PCR were performed using 2.5 U of KOD polymerase (Toyobo, Japan). The amplified fragment was purified by agarose gel electrophoresis and

inserted into the *Sma*I site of pUC19 (Takara, Japan), giving pUC-EFP. In a similar way, *Coniochaeta* FPOX cDNA was inserted into the *Eco*RI site of pKK223-3 (Amersham Pharmacia Biotech) using an upstream primer (primer 19) and a downstream primer (primer 20), giving pKK-CFP. Sequence analysis of the inserted region was carried out by the dideoxy chain termination method.

Expression and purification of recombinant FPOX-E and -C. The plasmid carrying the cDNA of FPOX-E or -C was introduced into *E. coli* JM109 cells and the transformants were cultured in 10 L of LB medium containing 50 μg/ml ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Then, the cells were harvested by centrifugation and FPOX-E or -C was purified by the same procedure used for native FPOXs from fungi cells.

Enzymatic measurement of glycated peptide. N^{α}-fructosyl Val-His-Leu-Thr-Pro-Glu (Fru-hexapeptide; Peptide Institute, Japan) dissolved in water at several concentrations was used as the standard solution. Five microliters of the standard solution and 5 μl of *Aspergillus oryzae* protease (20 mg/ml; Kikkoman, Japan) in 50 mM potassium phosphate buffer (pH 6.5) were incubated at 37 °C for 2 h. After heat inactivation (90 °C, 5 min), 140 μl of reaction mixture was added, incubation was continued at 30 °C for 20 min, and then the absorbance of the solution at 555 nm was measured. The reaction mixture contained 0.1 M potassium phosphate, pH 8.0, 0.5 mM TOOS, 900 U/L peroxidase, 0.45 mM 4-aminoantipyrine, and 0.1 U/ml FPOX-E or -C in a final volume of 150 μl. In addition, bacterial FAOX (Kikkoman, Japan) and fungal FAOX (ketoamine oxidase; Asahi Kasei, Japan) were used for the control experiments.

Results

Purification of FPOXs from E. terrenum and Coniochaeta sp.

FPOX was purified from the cell extract of *E. terrenum* with Fru-ValHis (Table 1). The purified FPOX from *E. terrenum* (FPOX-E) has a specific activity of 3.6 U/mg for the substrate Fru-ValHis. FPOX from *Coniochaeta* sp. (FPOX-C) was also purified from the cell extract by a similar process and its specific activity was 24 U/mg. The molecular mass of the enzyme subunit was estimated by SDS–PAGE to be 50,000 for FPOX-E and 52,000 for FPOX-C. G3000SWXL gel filtration showed that the molecular mass of the protein was 50,000 for FPOX-E and 60,000 for FPOX-C. From these results, the enzymes were considered to be monomeric.

Molecular cloning of FPOX cDNAs from E. terrenum and Coniochaeta sp.

Peptide sequences that were identified from the protease digestion of purified FPOX-E and -C are presented in Table 2. To initiate the cloning of FPOX-E, a PCR approach was taken (see "Materials and methods"), which resulted in the generation of a 924-bp PCR product. Several of the peptides identified from the peptide sequencing were found encoded in the 924-bp product, indicating that a partial cDNA encoding the FPOX-E had been obtained. Based on the partial cDNA for FPOX-E, several primer pairs were designed and used in 3′ and 5′ RACE to obtain sufficient amounts of specific PCR products for sequencing. The sequences of the 3′ and 5′ RACE fragments were connected to the 924-bp product by overlap to deduce the full length of the cDNA for FPOX-E. The sequence was 1314-bp long from the start codon (ATG) to the stop codon (TGA), which encodes a 437 amino acid polypeptide. (Fig. 5). The cDNA clone of FPOX-C was obtained using essentially the same method. The cDNA consisted of 1314-bp, which was of the same length as that of *Eupenicillium* cDNA. The deduced amino acid sequence of FPOX-C showed 76% identity with that of FPOX-E (Fig. 5). The cDNA sequences of FPOX-E and FPOX-C have been submitted to the DDBJ Nucleotide Sequence Database (Accession Nos. AB116146 and AB116147, respectively).

Table 2
Peptide sequences from the protease digestion of FPOX-E and -C

Peptide		Amino acid sequence
FPOX-E	Seq 1	TNVWLESR
	Seq 2	LHQPYGA
	Seq 3	PTDTYP
	Seq 4	NFILA
	Seq 5	LPNIG
	Seq 6	DLAEMPGW
FPOX-C	Seq 1	TIIAWLDNEDRIL
	Seq 2	QDGGWLAAAKAINAIQQFLK
	Seq 3	DKELFNR
	Seq 4	NFILATGDSGHSF
	Seq 5	HVVELIEGRLPEEMAYQWR
	Seq 6	APPKDLADMPGWKIT

Expression of FPOX cDNAs in E. coli

The plasmid carrying FPOX-E or FPOX-C cDNA was introduced into *E. coli* JM109. The cells were disrupted and the extracts were assayed for FPOX activity using Fru-ValHis as a substrate. JM109 [pUC-EFP] and JM109 [pKK-CFP] showed FPOX activity, while

Table 1
Summary of the purification of FPOX from *Eupenicillium terrenum* ATCC 18547

	Total activity (U)	Total protein (mg)	Specific activity (U/ml)	Purification (fold)	Yield (%)
Cell-free extract	0.765	298	0.00257	1	100
Ammonium sulfate (40–60% sat.)	0.780	71.6	0.0109	4.2	100
Ultrogel AcA34	0.643	6.69	0.0961	37	84
Q Sepharose FF	0.507	0.178	2.85	1100	66
POROS PE	0.0612	0.017	3.59	1400	8

Table 3
Primers used in polymerase chain reactions

(1) FPOX-E forward	5′-ACNAAYGTNTGGCTNGARWSNG-3′
(2) FPOX-E reverse	5′-KCCANCCNGGCATYTCNGC-3′,
(3) FPOX-C forward	5′-TGGYTNGAYAAYGARGAYGARAT-3′
(4) FPOX-C reverse	5′-TTRAARTTRTGICCRAARTCICCIGT-3′
(5) FPOX-E 5′ RACE	5′-CATCCCACAGATACCTACCCT-3′
(6) FPOX-E 5′ RACE anchor	5′-TCCCGATGTTTGGCAACAGC-3′
(7) FPOX-E 5′ RACE 1st forward	5′-GTGCCTGTGGTCTATGATGGTG-3′
(8) FPOX-E 5′ RACE 1st reverse	5′-CTCCACGCACCAGCAGCCAAGACAACCTTG-3′
(9) FPOX-E 5′ RACE 2nd forward	5′-ATCAACCGTACGGGGCTGCA-3′
(10) FPOX-E 5′ RACE 2nd reverse	5′-GATCGCATTGATAGCCTTGGC-3′.
(11) FPOX-C 3′ RACE	5′-CCCACAGACACTTATCCAGA-3′
(12) FPOX-C 5′ RACE anchor	5′-ACTCAGCGGCCTCTT-3′.
(13) FPOX-C 5′ RACE 1st forward	5′-AGATGGTACCAAATATTACGCTGACAAG-3′
(14) FPOX-C 5′ RACE 1st reverse	5′-TTTACACCACGTTCTTTCAAGAACTGT-3′
(15) FPOX-C 5′ RACE 2nd forward	5′-AAGCCTTGGGTGTATGCTCATATTCA-3′
(16) FPOX-C 5′ RACE 2nd reverse	5′-TCCTTGTATTTGGTCACGTTGAAGCAA-3′
(17) FPOX-E expression forward	5′-GACATGGCTCATTCGCGTGCAAGC-3′
(18) FPOX-E expression reverse	5′-CAAGAATCACAAATGTGCATCATGC-3′
(19) FPOX-C expression forward	5′-ATGACGTCGAATCGTGCAGATAC-3′
(20) FPOX-C expression reverse	5′-TTACAATTTCGGATCATGTTTCCAT-3′

JM109 [pUC19] and JM109 [pKK223-3] did not, implying that both FPOXs were expressed in active forms in *E. coli*. The FPOX activity in the cell extracts of JM109 [pUC-EFP] and JM109 [pKK-CFP] was 0.01 and 4.7 U/ml, respectively.

Properties of recombinant FPOX-E

The FPOXs from JM109 [pUC-EFP] and JM109 [pKK-CFP] were purified from the cell extract as described in Materials and methods. The purified preparations showed single bands on SDS–PAGE, indicating an apparent homogeneity of the proteins. The purified FPOX-E and FPOX-C had a specific activity of 5.43 and 23.8 U/mg of protein, respectively. The optimum pH for the enzyme activity of FPOX-E and FPOX-C was between 7.5 and 8.0 (Figs. 1A and B). Both enzymes were most active between 30 and 45 °C (data not shown). Incubation at 55 °C for 10 min resulted in complete inactivation of both enzymes (Figs. 2A and B). Enzyme activities were greatly reduced at pH values below 5.5 and above 9.5 (data not shown). In pH and temperature properties, the recombinant proteins were indistinguishable to the native proteins purified from fungi.

Substrate specificities of recombinant FPOXs

To investigate the substrate specificity of FPOXs, the enzyme assay was performed with varying concentrations of substrate. A typical Michaelis–Menten curve was obtained either when FPOX-E activity was measured with Fru-ValHis and Fru-Val (Fig. 3A) or when FPOX-C activity was measured with Fru-ValHis, Fru-Val, and εFru-Lys (Fig. 3B). Apparent K_m values of

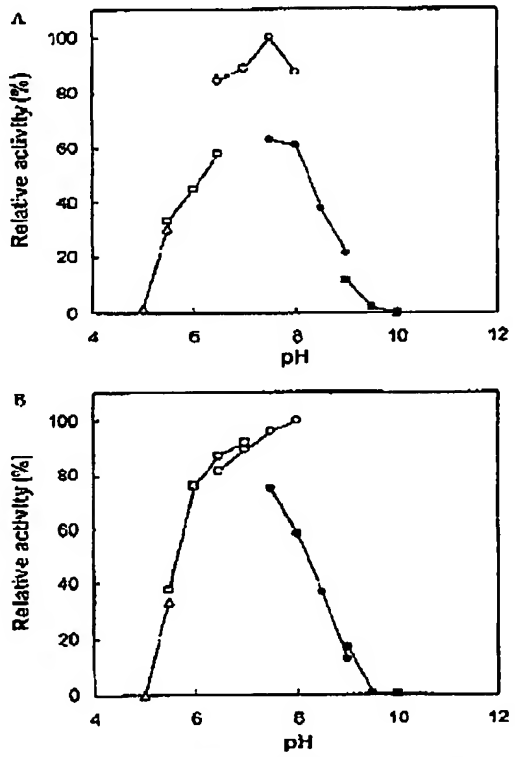

Fig. 1. Effects of pH on the activities of FPOX-E (A) and FPOX-C (B). Enzyme activities were measured in a standard reaction mixture in various buffers at 100 mM. Buffers: (△) acetate; (□) Mes–NaOH; (○) phosphate; (●) Tris–HCl; and (■) Ches–NaOH.

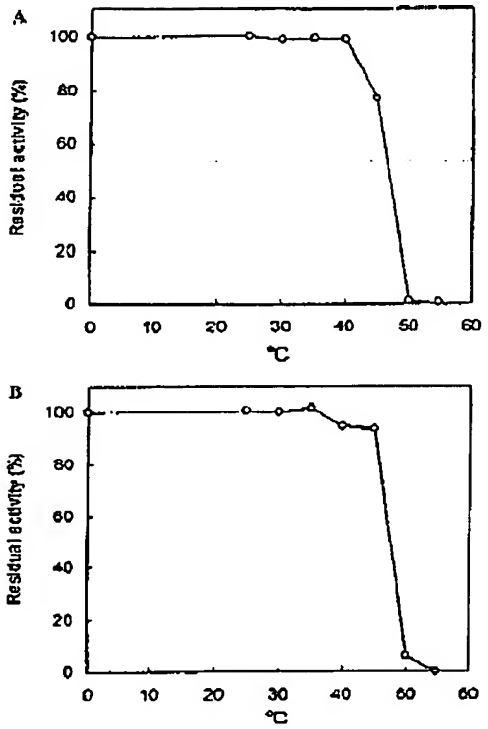

Fig. 2. Thermostability of FPOX-E (A) and FPOX-C (B). The activity remaining was measured after the purified enzyme was incubated in 100 mM potassium phosphate buffer (pH 8.0) at various temperatures for 10 min.

FPOX-E were 2.76 mM for Fru-ValHis and 0.318 mM for Fru-Val (Table 4), indicating high specificity toward Fru-Val. FPOX-C was also active toward εFru-Lys, although FPOX-E had quite low activity against εFru-Lys (Figs. 3A and B). Apparent K_m values of FPOX-C were 2.81 mM for Fru-ValHis, 0.824 mM for Fru-Val, and 10.6 mM for εFru-Lys, indicating also high specificity toward Fru-Val (Table 4).

Enzymatic measurement of glycated hexapeptide

HbA_{1c} is measured with the β-N-terminal hexapeptide (Fru-hexapeptide), which is released by enzymatic cleavage of the intact glycated hemoglobin molecule with endoproteinase Glu-C, using HPLC-ESI/MS or HPLC-CE [14]. For the enzymatic determination of Fru-hexapeptide, protease-treated Fru-hexapeptide was used as substrate for the FPOX reaction. As shown in Fig. 4, a linear relationship existed between the concentrations of protease-treated Fru-hexapeptide and the absorbance for FPOX reactions. A number of proteases

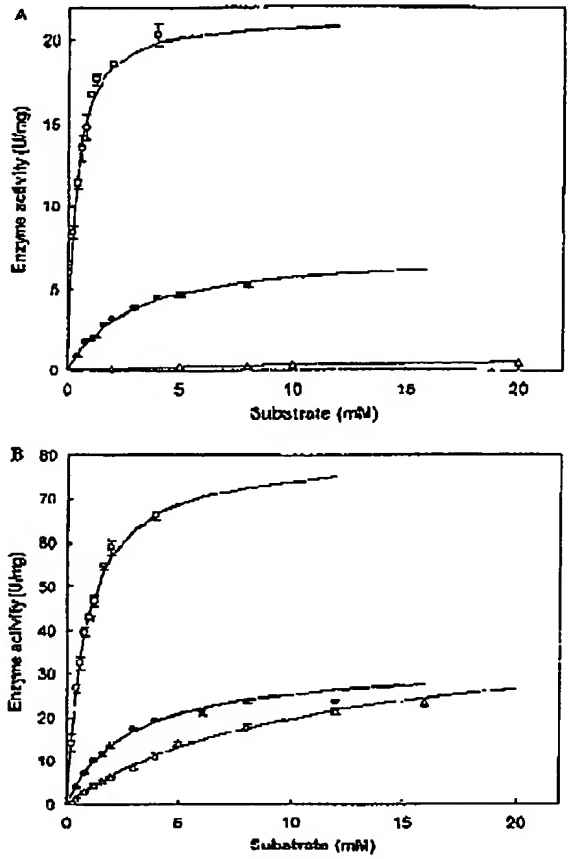

Fig. 3. Michaelis–Menten plots of FPOX-E (A) and FPOX-C (B) for glycated molecules. The plots were obtained by the standard reaction of FPOXs with different concentrations of N^{α}-fructosyl valine (□), N^{α}-fructosyl valyl-histidine (●), and N^{ε}-fructosyl lysine (△).

were tested in the reaction, and the *Aspergillus* protease was found to be one of the most effective for liberating substrate for FPOXs from Fru-hexapeptide. Since no absorbance was detected in the reaction of bacterial FAOX and fungal FAOX, FPOX activity toward fructosyl peptide was suggested to be indispensable for the enzymatic measurement of glycated hexapeptide.

Sequence similarity of FPOXs and FAOXs

A sequence alignment of FPOXs with existing FAOXs and other related proteins is shown in Fig. 5. The N-terminal AMP-binding motif, GxGxxG, and FAD attachment residue, Cys342 [15], were conserved in all proteins. Among six FPOX homologs, FAOD-P and

Table 4
Properties of N^{α}-fructosyl valine oxidases and N^{ϵ}-fructosyl lysine oxidases

	Molecular mass		Specific activity (U/mg)					Michaelis constant (mM)				
			α-Glycated molecule			ε-Glycated molecule		α-Glycated molecule			ε-Glycated molecule	
	Gel filtration	SDS-PAGE	Fru-Val	Fru-ValHis	αFru-εZLys	αFru-Lys	εFru-Lys	Fru-Val	Fru-ValHis	αFru-εZLys	αFru-εZLys	εFru-Lys
Fru-Val oxidase												
FPOX-C	60	52	66.0	23.8		23.4	0.824	2.91				10.6
FPOX-E	50	50	20.6	5.43		0.42	0.318	2.76				
FAOX-P [6]	39	49	18.6		18.6					0.62	0.19	
εFru-Lys oxidase												
FAOX-A [6]	106	51	9.62		9.62	32.0				0.51	0.37	
FLOD S-154 [3]	45	50	<0.01		<0.1		48.9			1.33	0.22	0.22
FLOD TF0937 [17]	48	47	3				30				0.194	

Z, benzyloxycarbonyl.

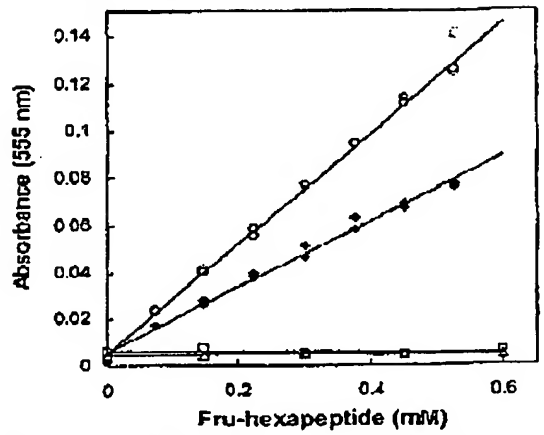

Fig. 4. Enzymatic measurements of Fru-hexapeptide with FPOXs. Fru-hexapeptide solutions at various concentrations were used for the protease-coupled FPOX activity. Symbols: (○) FPOX-C; (◆) FPOX-E; (△) bacterial FAOX; and (□) fungal FAOX.

FaoA showed striking similarity (70–85%) with FPOXs. On the other hand, FAOD-A, Amadoriase II, Amadoriase I, and FLOD showed low levels of similarity to FPOXs (30–40%), and their sequences were very similar to each other (≧50%). From these results, FPOXs and their homologs could be divided into two groups, the upper and lower groups in Fig. 5. This is reasonable given the substrate specificity of the enzymes, because the enzymes in the upper group (including FPOXs) had a similarly high level of activity against α-glycated molecules (e.g., Fru-Val), while those of the lower group had high levels of specificity toward ε-glycated molecules (e.g., εFru-Lys) (Table 4).

Discussion

Recently we have screened for a novel fructosyl peptide oxidase, an enzyme that could be used for the measurement of glycated hemoglobin levels in diabetic subjects with hyperglycemia [13]. Fructosyl peptide oxidases have been found in strains of eight genera: *Achaetomiella*, *Achaetomium*, *Chaetomium*, *Coniochaeta*, *Eupenicillium*, *Gelasinospora*, *Microascus*, and *Thielavia*. By using a purified FPOX from *Achaetomiella virescens*, we have shown that Fru-ValHis was consumed and the same molar amount of valyl-histidine was produced by the FPOX reaction [13]. The properties of the FPOXs characterized in this study are summarized in Table 4. FPOX-E and -C showed higher specificity toward Fru-Val than Fru-ValHis, however the activity toward Fru-ValHis is novel and particularly applicable to the enzymatic measurement of HbA_{1c}

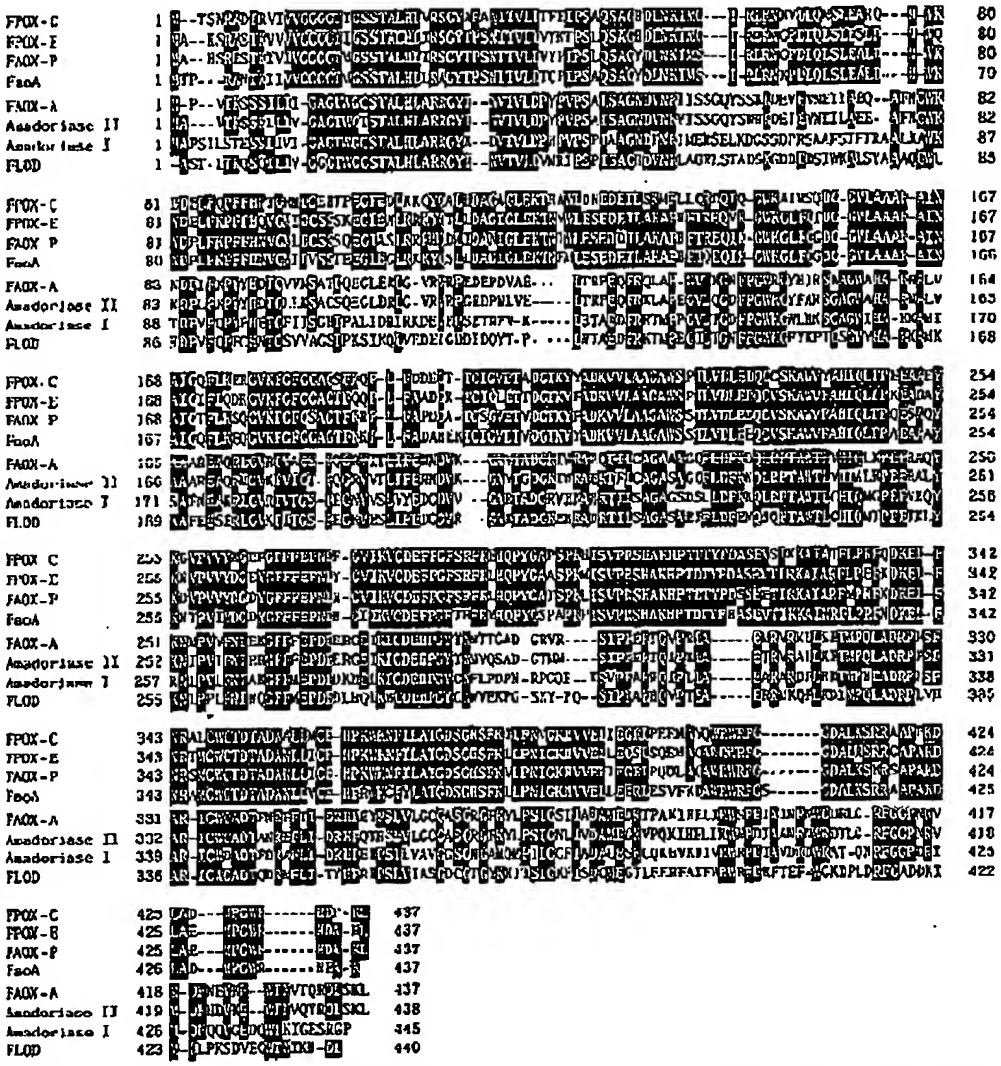

Fig. 5. Multiple sequence alignment of deduced amino acid sequences of FPOX-C, FPOX-E, and their homologs. Amino acid residues conserved in at least three of the four sequences in each group (upper, Fru-Val oxidases; lower, εFru-Lys oxidases) are shaded. Gaps (indicated by dashes) were appropriately introduced for optimal alignment. The residues of the AMP-binding motif and FAD attachment site of Cys are marked by asterisks. FPOX-C, FPOX from *Coniochaeta* sp.; FPOX-E, FPOX from *E. terreum*; FAOX-P, FAOX from *Penicillium funiculosum* [6]; FaoA, FAOX from *Aspergillus nidulans* [16]; FAOX-A, FAOX from *Aspergillus terreus* [6]; Amadoriase II, I, Amadoriase from *Aspergillus fumigatus* [11,15]; and FLOD, *N*ε-fructosyl lysine oxidase from *Fusarium oxysporum* IFO 9972 [17].

(discussed below). It is interesting that no FPOX activity was observed in FAOXs in spite of high sequence similarity toward FPOXs (Fig. 5). This difference in substrate recognition is thought to be determined by the substrate binding site of the enzymes. To reveal this mechanism of substrate recognition, cassette mutagenesis of FPOXs and FAOD-P or FaoA would provide important information.

FPOX-E and -C showed higher activity and lower K_m values toward Fru-Val than εFru-Lys (Table 4). And FAOX-P also showed that V_{max}/K_m for αFru-εZLys was much higher than that for εFru-αZLys [6], indicating that these three enzymes had higher specificity toward α-glycated molecules than ε-glycated molecules. In contrast, FAOX-A, FLOD S-1F4, and FLOD IFO9972 showed lower activity and/or higher K_m values toward α-glycated molecules (such as Fru-Val and αFru-εZLys) than ε-glycated molecules (such as εFru-Lys and εFru-αZLys), indicating high specificity toward ε-glycated molecules. Therefore, we categorized FPOXs and their homologs into two groups: (A) enzymes that preferably oxidize α-glycated molecules and (B) enzymes that preferably oxidize ε-glycated molecules (formerly described as fructosyl lysine oxidases [7]) (Table 4). A comparison of amino acid sequences revealed that these two groups (A and B) corresponded to the two groups that show high sequence similarity (Fig. 5). Therefore, they could be described as Fru-Val oxidases and εFru-Lys oxidases, and our novel FPOXs belonging to the former group. This relationship between sequence similarities and substrate specificities seemed reasonable, however further studies are needed to confirm this speculation.

FPOXs could be a useful diagnostic tool for determining the amount of HbA_{1c}. We are currently developing an enzymatic assay system for HbA_{1c} using FPOXs, since there are several proteases that are able to generate fructosyl peptides quite efficiently. In the present study, we have cloned, purified, and characterized two FPOXs; one from *Eupenicillium* and one from *Coniochaeta*. Both enzymes have strong activity toward Fru-Val and Fru-ValHis, however they differed in specificity toward ε-glycated molecules; FPOX-C acted on εFru-Lys, while FPOX-E showed slight activity toward εFru-Lys. Since Fru-hexapeptide is reported to be liberated from HbA_{1c} by Glu-C endoproteinase [14], we examined the enzymatic measurement of Fru-hexapeptide using FPOXs. As shown in Fig. 4, a linear relationship was obtained between the concentration of protease-treated Fru-hexapeptide and the absorbance for the FPOX reaction. The absorbance was not detected when we used bacterial FAOX and fungal FAOX (neither enzyme showed activity toward fructosyl peptide), indicating that not Fru-Val but Fru-ValHis was released by the protease treatment.

In this study, it was suggested that both FPOX-E and -C were available for assaying Fru-hexapeptide. However, FPOX-E could be a better candidate for the assay of HbA_{1c}, because internal Lys residues of proteins were glycated when the glucose level was high, and so εFru-Lys could become a major contaminant of the assay system. As FPOX-E acts mainly against Fru-ValHis even in the presence of εFru-Lys, it should provide for a precise assay of HbA_{1c}. Study is in progress to establish a rapid and simple enzymatic assay system for HbA_{1c} using FPOX-E.

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Nonenzymatic Glycosylation of Albumin *in Vivo*

IDENTIFICATION OF MULTIPLE GLYCOSYLATED SITES*

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Niggi Iberg and Rudolf Flückiger‡

From the Department of Research, Diabetology, University Clinics Basel, CH-4031 Basel, Switzerland

Nonenzymatic glycosylation of albumin *in vivo* occurs at multiple sites. Glucose gets attached to Lys-199, Lys-281, Lys-439, and Lys-525 as well as to some other lysine residues. The principal glycosylated site is Lys-525. Approximately 33% of the overall glycosylation occurs at this site. This site specificity is remarkable and is postulated to be a consequence of local catalysis of the nonenzymatic glycosylation reaction. It appears that positively charged amino groups in the protein catalyze the Amadori rearrangement at specific sites. The principal glycosylated site, Lys-525, lies in a Lys-Lys sequence; other glycosylated sites lie in a Lys-Lys, Lys-His, and Lys-His-Lys sequence or are near disulfide bridges, which are likely to place amino groups of more remote parts of the protein closer to these sites. The occurrence of nonenzymatic glycosylation at most of the identified sites in albumin from diabetic patients is explained by the concept of local acid-base catalysis of the Amadori rearrangement.

Enhanced nonenzymatic glycosylation of proteins has been claimed to be of relevance in the development of some complications in diabetes mellitus (1–3). Because the glycosylation reaction is nonenzymatic in nature, it has been expected to occur generally with all kinds of proteins, but preferentially at primary amino groups with a low pK value. Although the occurrence of this reaction with a variety of proteins *in vivo* has been demonstrated, its apparent specificity has remained unexplained. The prediction that the reaction should occur at amino groups with a low pK value is supported by one example only: the glycosylation of the NH_2 terminus of the β-chain in hemoglobin which yields HbA1c (4). In both albumin (5–7) and hemoglobin (4), glycosylation generally occurs at sites irrespective of their pK value.

In RNase A, which was incubated with glucose, glycosylation was also found to occur at lysine residues irrespective of their pK value (8).[1] It was realized that glycosylation occurs predominantly at lysine residues which are close to another amino group, and we hypothesized that acid-base catalysis of the Amadori rearrangement (9) by these close amino groups causes site specificity.[1]

In this report we describe the results of our attempt to validate this concept for the *in vivo* glycosylation of proteins by elucidating the glycosylated sites in albumin. In addition to the principal glycosylated site in albumin identified by Garlick and Mazer (7) we identified several minor glycosylated sites. All these sites are likely to be close to charged amino groups that can act catalytically.

EXPERIMENTAL PROCEDURES

Materials—The affinity support Blue Trisacryl was a product of LKB (Bromma, Sweden), and the boronate affinity support Affi-Gel 601 was from Bio-Rad. The gel filtration resins Sephadex G-15, Sephacryl S-200, and Trisacryl GF-05 were obtained from Pharmacia (Uppsala, Sweden) and LKB (Bromma, Sweden), respectively. [^{3}H]Sodium borohydride was purchased from Amersham (Amersham, Buckinghamshire, UK).

Isolation of Albumin—Blood was obtained from a diabetic patient, in poor glycemic control, and anticoagulated with EDTA. The plasma was collected and stored at −70 °C until used. Before application to the affinity column, 3-ml sample aliquots were filtered and passed over a Trisacryl GF-05 column, equilibrated with the application buffer of the affinity column. The plasma proteins were then applied to a column (diameter 1.6 cm) packed with 45 ml of Blue Trisacryl (10, 11), equilibrated with application buffer (0.05 M Tris/HCl, 0.5 M NaCl, pH 8.0), and the column was washed with 75 ml of application buffer at a flow rate of 30 ml/h. The adsorbed albumin was eluted with 2.5 M NaCl in 0.05 M Tris/HCl. Albumin from three runs was combined and concentrated to a volume of 16 ml in an Amicon ultrafiltration system. This yielded 330 mg (5 μmol) of albumin.

Labeling of the Glycosylated Sites—In order to label the glycosylated sites, the ketoamine adduct in albumin was reduced with ^{3}H-labeled $NaBH_4$ (12). A 100-fold excess of radioactive $NaBH_4$ (20 mCi/mmol) was slowly added and the reaction allowed to proceed for 1 h on ice at pH 8.0. The reaction was stopped by the addition of acetic acid to a pH of 3.0.

Denaturation, Disulfide Cleavage, and Alkylation—Guanidinium/HCl and Tris/HCl were added to the above solution to yield a volume of 50 ml and a concentration of 6 M guanidinium/HCl and 0.5 M Tris/HCl. The pH was adjusted to 8.2. After 30 min at 50 °C, 150 mg of dithiothreitol (5 mg/10 mg albumin) were added and disulfide cleavage allowed to proceed for 1 h (13). The solution was cooled to room temperature, 750 mg of iodoacetate (25 mg/10 mg albumin) were added (14), and after 1 h in the dark dialyzed extensively against 0.5% ammonium acetate. The solution was concentrated to 12 ml by Amicon ultrafiltration. 240 mg of alkylated and labeled albumin were obtained.

In order to isolate monomeric albumin, approximately 120 mg of the above material were passed over a column (diameter 2.2 cm) packed with 300 ml of Sephacryl S-200.

Tryptic Digestion—Tryptic digestion was performed with 90 mg of albumin. To destroy potential peptidase activity, the albumin solution was heated for 5 min to 100 °C. 1.6 mg of trypsin, purified by passage over Glycogel B, was added. Digestion was allowed to proceed for 8 h at 37 °C and stopped by briefly heating to 100 °C. Experience showed that during digestion turbidity developed. This insolubility does not occur if digestion is performed in 2 M guanidinium/HCl in 0.5% ammonium acetate, pH 8.0 (15). The solution of tryptic peptides showed no turbidity nor was there any precipitate.

Isolation of the Tryptic Peptides—The glycosylated and labeled tryptic peptides were isolated by boronate affinity chromatography (16). The tryptic digest was applied to a column (diameter 2.2 cm) packed with 7 ml of Affi-Gel 601, equilibrated with application buffer

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‡ Recipient of a grant from the Johnson Foundation. To whom correspondence should be addressed: Departement Forschung, ZLF, Kantonsspital Basel, Hebelstrasse 20, CH-4031 Basel, Switzerland.

[1] N. Iberg and R. Flückiger, manuscript in preparation.

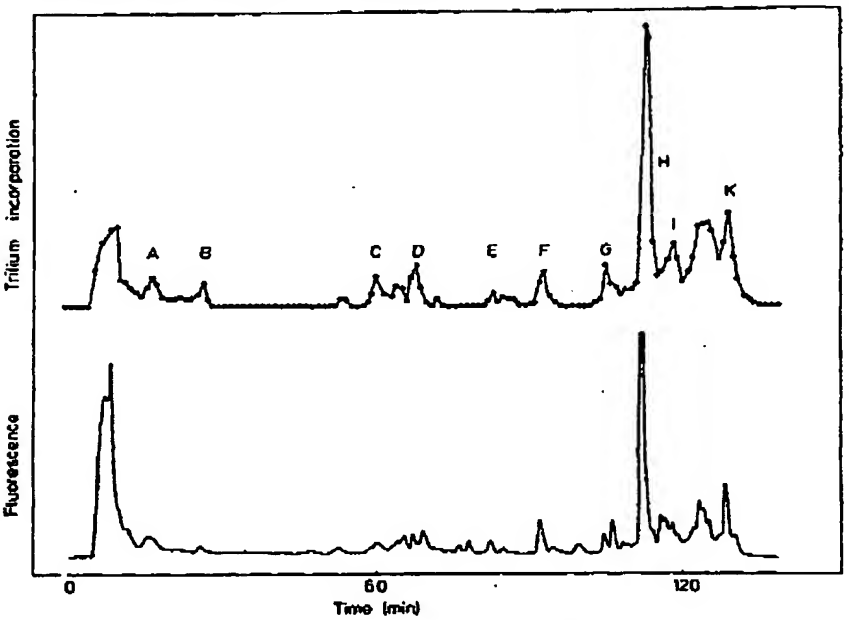

FIG. 1. **Separation of glycosylated and labeled peptides from albumin on reverse-phase HPLC.** The glycosylated peptides were separated by HPLC as detailed under "Experimental Procedures." The eluent was monitored for radioactivity by liquid scintillation counting (*upper panel*) and for peptides by fluorescence with a fluorescamine detection system (*lower panel*).

(0.1 M ethylmorpholine, 2 M guanidinium/HCl, pH 9.0). For application, the 26 ml of digest were circulated over the column for 4 h at a flow rate of 30 ml/h. The column was washed with 100 ml of application buffer, and the adsorbed glycosylated peptides were eluted with 0.2 M sorbitol in the application buffer.

It is noteworthy that about 50% of the radioactivity did not bind to the affinity support. This radioactivity represents nonspecific incorporation, because the unbound material did not bind when reapplied. The nature of such nonspecific side reactions has been investigated. It originates from superoxides and radicals which are formed by decomposition of radioactive $NaBH_4$. These stabilized radicals lead to the reddish appearance of the commercial product.[2]

Separation of the Glycosylated Peptides—The glycosylated peptides were separated by HPLC,[3] using the reverse-phase column Aquapor RP-300 (Brownlee) and a detection system for peptides with fluorescamine (17). The column was operated at a flow rate of 50 ml/h. A gradient with buffer A (5% methanol in 0.025 M trifluoroacetic acid) and buffer B (75% methanol in 0.025 M trifluoroacetic acid) was used. For the first 15 min the column was run isocratically in buffer A, followed by 60 min linear gradient elution up to 35% buffer B, 15 min isocratic elution at 35% buffer B, and finally by 30 min up to 100% buffer B.

Lyophilyzed peptides dissolved in 2 ml of buffer A, corresponding to 2.5 mg of albumin, were applied and fractions of 1 min collected. Radioactivity was measured by liquid scintillation counting (Fig. 1). The pooled fractions with the glycosylated peptides were lyophilyzed. The estimated amount of the peptide corresponding to the principal glycosylated site (peak H) was 4–40 nmol.

Hydrolysis and Amino Acid Analysis—The composition of the isolated glycosylated peptides was determined by amino acid analysis after hydrolysis in 6 M HCl under N_2 at 110 °C for 24 h. Hydrolysates were lyophilyzed, dissolved in 0.1% acetic acid, and lyophilyzed a second time. Amino acid analysis was performed on the Pico Tag System from Waters (18). An estimated 100 pmol of amino acids of peak H, and about 10 pmol of the other peaks, were analyzed.

RESULTS

Sequence assignment, based on the compositional data, was attempted by the following criteria. 1) It was assumed that tryptic digestion was complete, *i.e.* that all the radioactive peaks represented two tryptic peptides not split at the glycosylated lysine residue. 2) Mismatching "dipeptides" were excluded based on the reliably determined amino acids: Glu, Ala, Leu, Phe, Lys, Cys, and occasionally also Asp and Pro. 3) Positive sequence assignment required that every reliably determined amino acid in the peptide was detected in approximately the expected amount. "Noise" up to 0.5 residues was tolerated. By these criteria unequivocal assignment was possible for the compositional data of four peaks (Table I).

Due to the small amount of peptides of the minor glycosylated sites, there was considerable noise, and assignment and quantitation was not equally reliable for all peaks. Six other peptides could be assigned if more variability was accepted (Table II). Some radioactive peaks could not be assigned. These nonassigned radioactive peaks correspond to poorly

TABLE I

Amino acid composition of the definitely assigned glycosylated tryptic peptides of albumin

Peak	A		D		F		H	
Sequence	427–444		196–205		277–286		525–534	
Glycosylated site	Lys-439		Lys-199		Lys-281		Lys-525	
Amino acid	Composition (theoretical and experimental data)							
Glu	1	1.0	1	1.3	3	2.6	2	2.4
Ser		0.8	1	0.9				
His	1	+						
Thr							1	1.0
Ala	1	0.8	1	1.0			1	1.1
Pro	1	0.7		0.7	1	1.1		
Tyr		0.5						
Val		0.5					2	2.0
Cys[a]	2	+	1	+	2	+		
Leu			2	1.7	2	2.5	2	2.0
Lys	1	1.0	1	0.9	1	0.8	1	1.0
Glc-Lys[a]	1	+	1	+	1	+	1	+

[a] The elution position of alkylated Cys and Glc-Lys were determined by comparison with appropriate standards (13, 19).

[2] R. Kissner, personal communication.

[3] The abbreviation used is: HPLC, high performance liquid chromatography.

TABLE II

Amino acid composition of the other glycosylated tryptic peptides

Peak	B	C	E	G	I	K
Sequence	226–240	314–323	349–359	11–20	525–536	226–240
Glycosylated site	Lys-233	Lys-317	Lys-351	Lys-12	Lys-534	Lys-233
Amino acid	Composition (theoretical and experimental data)					
Asp	1 1.6	2 1.6	1.5	2 1.1	1.2	1 1.3
Glu	2 2.1	1 1.5	3 2.5	2 1.8	2 2.2	2 2.4
Ser	1 1.0		1.2	0.4	0.6	1 0.7
Gly				1 1.3		
His					1 +	
Thr	2 0.6		2 1.4		1 0.7	2 0.8
Ala	2 1.6	2 1.4	1 1.2		1 1.4	2 1.6
Tyr		1 1.3	1 1.7	0.7	0.5	0.6
Val	2 1.3	1 0.9		0.5	2 1.3	2 1.5
Cys*		1 +			+	+
Leu	2 2.0	0.7	2 2.0	1 1.2	2 2.0	2 2.0
Phe	1 0.8			2 1.1	1 0.6	1 1.0
Lys	1 1.0	1 1.0	1 0.6	1 1.0	1 1.0	1 0.9
Glc-Lys*	1 +	1 +	1 +	1 +	1 +	1 +

* see Table I.

resolved peaks in the fluorescence monitoring. Interestingly, peaks B and K correspond to the same glycosylated site, a finding that remains unexplained.

DISCUSSION

The nonenzymatically glycosylated tryptic peptides of albumin were isolated by boronate affinity chromatography (16) and resolved by HPLC (Fig. 1). This separation showed that at least 10 different lysine residues out of a total of 59 get nonenzymatically glycosylated to some extent. Four glycosylated sites were clearly established: Lys-439, Lys-199, Lys-281, Lys-525. Evidence for the assignment of five other sites is less certain but consistent with glycosylation at Lys-233, Lys-317, Lys-351, Lys-12, Lys-534.

Glycosylation at Lys-525 accounts for approximately one-third of the overall glycosylation, confirming an earlier report on the principal glycosylated site in albumin (7). In this report five to six minor radioactive peaks were also resolved but the respective peptides not characterized.

Lys-199 is one of the other unequivocally assigned glycosylated lysine residues. Indirect evidence for glycosylation at Lys-199 had been derived from the observation that acetylsalicylic acid, which is known to acetylate Lys-199 (15), inhibits the nonenzymatic glycosylation of albumin *in vitro* (5). However, the extent of this inhibition, which was approximately 50%, cannot be explained by our data which show that glycosylation at Lys-199 accounts for only approximately 5% of total glycosylation.

Strikingly, despite its low pK of 7.9 (20), glycosylation at Lys-199 is only marginal. Glycosylation of the α-amino group of the NH_2-terminal asparagine, the other site with a low pK value, was not detectable. The pK value therefore seems to be of minor importance for the extent of glycosylation at specific sites.

We have postulated that structural features of the protein influence the extent of nonenzymatic glycosylation by way of catalysis of the Amadori rearrangement. Appropriately located positively charged amino acid residues could afford local acid-base catalysis. It is well established that acid-base catalysis enhances the Amadori rearrangement in simple model systems (9). Catalysis of the Amadori rearrangement as a possible explanation for site specificity has been previously considered (7, 8), but it was assumed that carboxylic groups would act as acid catalysts. However, at a physiological pH, the fully dissociated carboxyl group cannot donate a proton.

Considering the position of the glycosylated sites in albumin, it is striking that three sites are located in a sequence of basic amino acids: Lys-525 in a Lys-Lys sequence, Lys-439 in a Lys-His sequence, and Lys-534 in a Lys-His-Lys sequence. Other glycosylated sites in albumin are also likely to be neighbored by positively charged amino acid residues. Lys-199, Lys-281, Lys-317, and Lys-439 are close to disulfide bridges, which place a positively charged amino group in a more remote part of the sequence close to these sites.

For the remaining minor glycosylated sites, Lys-12, Lys-233, and Lys-351, there is no evidence for proximity to positively charged groups due to lack of knowledge of tertiary structure of albumin. Evaluating the validity of this concept with literature data on hemoglobin, we found the following supporting evidence (21): the principal glycosylated site, the amino terminus of the β-chain, is adjacent to a histidine and close to a lysine residue. Two other glycosylated sites, β-Lys-66 and α-Lys-61, are located in a Lys-Lys sequence (4) and some of the other minor glycosylated sites are within 5 Å of positively charged groups (22).

It has been found that glycosylation of hemoglobin *in vivo* and *in vitro* affects different sites (4). This observation has not been explained but may be a consequence of differing reaction conditions or partial denaturation of the protein. In albumin, partial denaturation may explain why the indirect evidence for glycosylation of Lys-199 from *in vitro* glycosylation did not reveal the principal site glycosylated *in vivo*.

In summary, the concept of local acid-base catalysis of the nonenzymatic glycosylation reaction explains the site specificity of this reaction in all known instances: albumin, hemoglobin, and RNase A.

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Purification and Properties of Fructosyl Lysine Oxidase from *Fusarium oxysporum* S-1F4

Yasuyoshi SAKAI, Nobuyuki YOSHIDA, Atsuko ISOGAI, Yoshiki TANI,* and Nobuo KATO**

Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606–01, Japan
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Fructosyl lysine oxidase (FLOD) was examined for its use in the enzymatic measurement of the level of glycated albumin in blood serum. To isolate micoorganisms having such an enzyme activity, we used N^{ε}-fructosyl N^{α}-Z-lysine (ε-FL) as a sole nitrogen source in the enrichment culture medium. The isolated fungus, strain S-1F4, showed a high FLOD activity in the cell-free extract and was identified as *Fusarium oxysporum*. FLOD was purified to an apparent homogeneity on SDS-PAGE. The molecular mass of the subunit was 50 kDa on SDS-PAGE and seemed to exist in a monomeric form. The enzyme had an absorption spectrum characteristic of a flavoprotein and the flavin was found to be covalently bound to the enzyme. The enzyme acted against N^{ε}-fructosyl N^{α}-Z-lysine and N^{α}-fructosyl N^{ε}-Z-lysine and showed specificity for fructosyl lysine residues.

Nonenzymatic glycosylation of proteins (glycation) proceeds by an initial condensation of reducing sugars such as glucose with α- or ε-amino groups of the proteins to form a Schiff's base. This labile aldimine adduct may either dissociate or undergo the Amadori rearrangement to produce a more stable ketoamine product.[1] Glycation causes the browning of foods during long-term storage, a problem in the food industry. *In vivo*, glycation of blood proteins, hemoglobin and albumin, was reported to be enhanced in patients with diabetes mellitus. As the amount of glycated proteins reflects the level of blood glucose in the recent several weeks and is not affected by transient increases of glucose in the blood,[2] the level of glycated protein is expected to be a good index of the medical condition of the patients.

At present, the amount of glycated hemoglobin and albumin are estimated by an HPLC method,[3] thiobarbituric acid assay,[4] aminophenylburonic acid affinity chromatography,[5] or fructosamine method.[6] The specificities of these methods are low and the assay is usually too tedious for examining many specimens. To overcome these demerits, we are developing an enzymatic method to measure the amount of glycated proteins in the serum. As the lysine residues of proteins are known to be most often glycated,[7–13] an enzyme catalyzing the H_2O_2-producing oxidase reaction where fructosyl lysine is deglycated is considered to be the most efficient for the measurement of glycated proteins. In this study, we describe the isolation of *Fusarium oxysporum* S-1F4, which produces a fructosyl lysine oxidase (FLOD), and the purification and characterization of FLOD is also described.

Materials and Methods

Materials. DEAE-Sephacel, Sephacryl S-200, and Mono Q were purchased from Pharmacia Fine Chemical, Uppsala, Sweden. Butyl-Toyopearl and phenyl-Toyopearl were from Tosoh Co., Tokyo, Japan. Fructosyl amino acids were a kind gift from Kyoto Daiichi Kagaku Co. Ltd., Kyoto, Japan. Horse radish peroxidase was from Sigma Co., St. Louis, U.S.A. Adenylate kinase was purchased from Unitika, Ltd., Osaka, Japan. Pronase was purchased from Calbiochem Co., California, U.S.A. Glucosone was prepared by the method of Becker *et al.*[14] DA-67 was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Screening for FLOD-producing microorganisms. The enrichment culture method was used to obtain the FLOD-producing microorganisms. The medium contained 5 g ε-FL, 10 g glucose, 1 g K_2HPO_4, 1 g NaH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1% (v/v) vitamin mixture, and 1.0% (v/v) metal solutions in 1000 ml of distilled water (pH 5.5). The vitamin mixture had the following composition; 1 mg thiamin·HCl, 2 mg riboflavin, 2 mg Ca-pantothenate, 2 mg pyridoxine·HCl, 0.1 mg biotin, 1 mg *p*-aminobenzoic acid, 2 mg nicotinic acid, and 0.1 mg folic acid in 100 ml of distilled water. The composition of the metal solutions was as follows; 1.7 g $MnSO_4 \cdot 3H_2O$, 2.2 g $ZnSO_4 \cdot 7H_2O$, 0.4 g, $CuSO_4 \cdot 5H_2O$, 0.28 g, $CoCl_2 \cdot 2H_2O$, 0.26 g $Na_2MoO_4 \cdot 2H_2O$, 0.4 g H_3BO_3, and 0.06 g KI in 1000 ml of distilled water. A soil sample was added to 3 ml of culture medium described above, and shaken (300 strokes per min) at 28°C for 2 days. Then 0.03 ml of the culture was transferred to fresh medium and the cultivation was done under the same conditions. Pure cultures were obtained by inoculation onto agar plates of the same medium. Single colonies were tested by the FLOD assay.

Assay of FLOD activity. FLOD activity was measured at 30°C by the formation of a quinone dye following the absorbance at 505 nm ($\varepsilon = 5.13 \times 10^3$) with a Shimadzu UV-160 spectrophotometer. The reaction mixture contained 100 μmol Tris–HCl (pH 8.0), 4.5 μmol 4-aminoantipyrine, 6.0 μmol phenol, 6.0 unit peroxidase, and 5.0 μmol ε-FL in a total volume of 3 ml. The reaction was linear from 5 to 10 min under these conditions. One unit of enzyme activity was defined as the amount of the enzyme that produced 0.5 μmol of quinone dye per minute. This amount corresponds to the formation of 1.0 μmol H_2O_2 per min.

Protein methods. Protein was measured by the method of Bradford[15] with a Bio-Rad Protein Assay Kit using bovine serum albumin as a standard. The molecular weight of FLOD was determined by gel filtration on a Sephacryl S-200 column equilibrated with 0.1 M Tris–HCl buffer (pH 8.0) containing 2 mM DTT and 0.1 M NaCl. The molecular mass of the subunits of the purified FLOD was measured by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli[16] with 10% gel (1 mm thick, 8 × 9 cm). Protein was stained with Coomassie Brilliant Blue R-250.

Purification of FLOD. The purification was done at 4°C. Of the SH-reagents tested, dithiothreitol (DTT) was the most effective in the

* Present address: *Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma 630–01, Japan.*
** Corresponding author.

Abbreviations: ε-FL, N^{ε}-fructosyl N^{α}-Z-lysine; α-FL, N^{α}-fructosyl N^{ε}-Z-lysine; α-ZL, N^{α}-Z-lysine; FLOD, fructosyl lysine oxidase; Z-, benzyloxycarbonyl-.

prevention of enzyme inactivation. NY buffer (50 mM Tris–HCl buffer, pH 8.5, containing 2 mM DTT) was used thoughout the purification procedure unless otherwise stated. Strain S-1F4 was cultivated in 10 liters of the medium described above using 0.2% yeast extract in place of vitamin and metal solutions at 28°C for 36 h.

1. Preparation of cell extract. Washed mycelia (200 g as wet weight) were suspended in 1 liter of 0.1 M Tris–HCl buffer (pH 8.5) containing 2 mM DTT, and disrupted by a Dyno-Mill (Willy A. Bachofen Manufacturing Engineers, Basel, Switzerland). The homogenate was centirufged at 9,000 × *g* for 30 min to remove unbroken cells and cell debris.

2. Ammonium sulfate fractionation (1st). Ammonium sulfate was added to the above supernatant to 40% saturation, stirred for 1 h, and centrifuged at 20,000 × *g* for 10 min. The supernatant, to which ammonium sulfate was added further to 75% saturation, was stirred for 1 h, and centrifuged at 20,000 × *g* for 10 min. The supernatant was removed and the resultant precipitate was dissolved in and dialyzed with NY buffer for 18 h under gentle stirring at 4°C.

3. DEAE-Sephacel column chromatography. The dialyzed solution was put on a DEAE-Sephacel column (4.2ϕ × 26 cm) equilibrated with NY buffer. The column was washed with the same buffer. The absorbed protein was eluted under a linear KCl gradient (0–0.5 M).

4. Ammonium sulfate fractionation (2nd). Active fractions were collected and ammonium sulfate fractionation was done as described above, and the precipitate obtained at the ammonium sulfate concentration between 55 and 75% saturation was dissolved in NY buffer and then dialyzed against NY buffer containing 25% saturation of ammonium sulfate.

5. Phenyl-Toyopearl column chromatography. The dialyzed enzyme solution was put on a phenyl-Toyopearl column (1.8ϕ × 30 cm) equilibrated with NY buffer containing 25% saturation of ammonium sulfate. Elution was done under a linear gradient of ammonium sulfate (25–0% saturation).

6. Butyl-Toyopearl column chromatography. Active fractions from the previous step were collected and precipitated by addition of ammonium sulfate to 80% saturation. The precipitate was dissolved and put on a butyl-Toyopearl column (2.0ϕ × 11 cm) equilibrated with NY buffer containing 40% saturation of ammonium sulfate. The absorbed protein eluted under a linear gradient of ammonium sulfate (40–0% saturation).

7. Sephacryl S-200 column chromatography. The active fractions obtained in step 6 were concentrated by ultrafiltration, and put on a Sephacryl S-200 column (1.7ϕ × 86 cm) equilibrated with 0.1 M Tris–HCl buffer (pH 8.5) containing 2 mM DTT and 0.1 M NaCl. Gel filtration was done with the same buffer.

8. Mono Q chromatography. The active fractions after gel filtration were collected and dialyzed by ultrafiltration, and put on a Mono Q HR5/5 column in the Pharmacia FPLC system. A gradient with NY Buffer, and NY2 buffer (NY buffer containing 0.5 M KCl) was used. For the first 5 min, the column was run isocratically with NY buffer, followed by a linear gradient up to 12.5% NY2 buffer for 28 min.

Flavin content. Flavin was identified and measured by the AMP liberated from the enzyme. Purified FLOD (6.29 nmol) in 0.1 M Tris-HCl buffer (pH 8.5) was incubated with 1 mg Pronase at 37°C for 20 h. After inactivation of Pronase in boiling water, 0.6 mU of phosphodiesterase was added and incubated at 37°C for 30 min. AMP in the reaction mixture was measured by the enzymatic method with adenylate kinase.[17]

Reaction products. ε-FL and N^{α}-Z-lysine (α-ZL) was measured by HPLC on an L-column ODS (4.6 × 150 mm; Chemical Inspection & Testing Institute, Tokyo, Japan) at 37°C with 50% KH_2PO_4 (20 mM) in acetonitrile as the solvent at a flow rate of 1.0 ml/min with monitoring of the absorbance at 254 nm. Glucosone was analyzed on HPLC with a Shimpack Ion KS-801 (8.0 × 300 mm; Shimadzu Co., Kyoto, Japan) at 60°C with distilled water as the solvent and detected with a Toyo Soda RI-8 refractometer. H_2O_2 was measured with peroxidase using DA-67 as the coloring reagent. Oxygen consumption was measured with an oxygen electrode system (Rank Brothers, Cambridge, England).

Results

Screening of the FLOD-producing microorganisms

About 300 cultures that grew on ε-FL as a sole nitrogen source were isolated from various soil samples. Among them, the highest FLOD activity was found in the cell-free extract of a fungus, strain S-1F4 (6.03×10^{-2} units/mg·protein), which was identified as *Fusarium oxysporum* by Centraalbureau voor Schimmelculture (Delft, The Netherlands).

Purification of FLOD

FLOD was purified 796-fold from the cell-free extract as

Table I. Summary of FLOD Purification

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell-free extract	443	7210	0.0614	1	100
Ammonium sulfate (40–75%)	412	3030	0.136	2.21	93
DEAE-Sephacel	264	184	1.44	23.5	60
Ammonium sulfate (55–75%)	260	72.3	3.60	58.6	59
Phenyl-Toyopearl	149	4.95	30.1	490	34
Butyl-Toyopearl	117	2.34	50.0	814	26
Sephacryl S-200	69.5	1.43	48.5	790	16
Mono Q	29.5	0.603	48.9	796	6.7

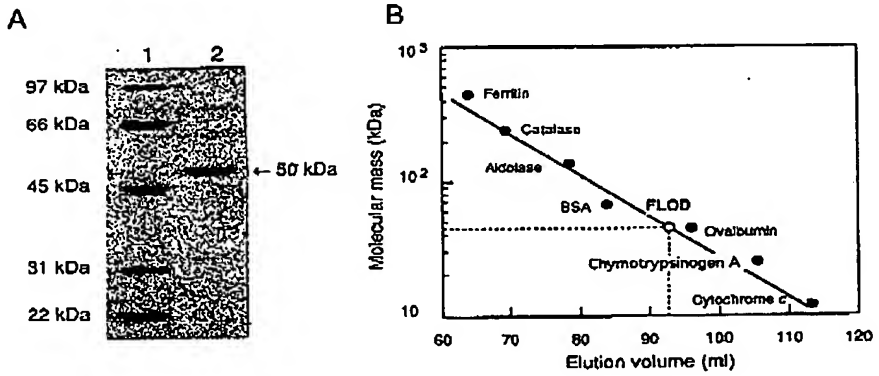

Fig. 1. Molecular Mass Estimation of FLOD.

A: SDS–PAGE pattern of FLOD. Lane 1: Marker proteins; Lane 2: Purified FLOD. Bio-Rad SDS–PAGE Molecular Weight Standards-Low was used as the molecular weight standards. B: Sephacryl S-200 gel filtration of FLOD. Mol-Ranger Molecular Weight Marker Kit (Pierce Chemical Company, Rockford, U.S.A.) was used as the molecular mass markers. Experimental conditions are described in Materials and Methods.

described in Materials and Methods (Table I). The purified FLOD has a specific activity of 48.9 units/mg of protein. The purified preparation showed a single band on SDS–PAGE, indicating an apparent homogeneity of the protein (Fig. 1). Specific activity was not increase by Sephacryl S-200 and Mono Q column chromatographies, but two more steps were necessary to remove some minor protein bands appeared on SDS–PAGE.

Molecular mass

The molecular mass of the enzyme subunit was estimated to be 50 kDa on SDS–PAGE. Sephacryl S-200 gel filtration showed that the molecular mass of FLOD was 45 kDa. From these data, the enzyme is considered to be monomeric (Fig. 1). The enzyme was found to contain no sugar chain from peroxidase coupled lectin blot analysis and no metal ion by atomic absorption spectrophotometry (data not shown).

Stoichiometry

The stoichiometry of the reaction catalyzed by FLOD was examined. The molar amount of oxygen consumed and that of α-ZL, glucosone, and H_2O_2 produced by the enzyme reaction was closely equivalent (Table II) indicating that the enzyme catalyzes an oxidase reaction as shown in Fig. 2.

Table II. Stoichiometry of the Reaction Catalyzed by FLOD

O_2 consumed (μmol)	α-ZL produced (μmol)	Glucosone produced (μmol)	H_2O_2 produced (μmol)
0.360	0.422	0.349	0.346

After 1 μmol of ε-FL was incubated with 1 U of FLOD at 30°C for 3 h, the amounts of oxygen, α-ZL, glucosone, and H_2O_2 were measured as described in Materials and Methods.

Substrate specificity

Table III shows the substrate specificity of FLOD. The enzyme was active toward N^{α}-fructosyl-N^{ε}-Z-lysine (α-FL) as well as ε-FL. FLOD did not catalyze the oxidation of D-glucose, D-fructose, and L-lysine. The activity for fructosyl valine was very low compared with those for ε-FL and α-FL. The enzyme reacted with fructosyl poly L-lysine, but not with poly L-lysine. In addition, the enzyme did not act against intact glycated protein such as glycated human serum albumin (Sigma Co., St. Louis, U.S.A.) (data not shown). Michaelis constants for ε-FL and α-FL were 0.22 mM and 1.33 mM, and maximal reaction velocities were 122 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ and 127 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, respectively.

Effects of temperature and pH on FLOD activity and stability

The optimum temperature and pH of the enzyme were

Table III. Substrate Specificity of FLOD

Substrate	Concentration in reaction mixture	Relative activity (%)
N^{α}-Fructosyl N^{ε}-Z-lysine	1.67 mM	100
N^{α}-Fructosyl N^{ε}-Z-lysine	1.67	103
Fructosyl valine	1.67	<0.1
N^{ε}-Methyl-L-lysine	1.67	N.D.*
N^{ε}-Fructosyl poly-L-lysine	0.02%	2.3
Poly-L-lysine	0.02	N.D.

* N.D., not detected.

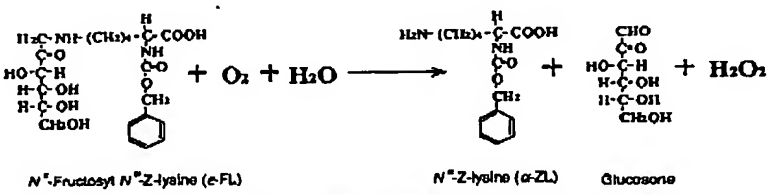

Fig. 2. The Reaction Catalyzed by FLOD.

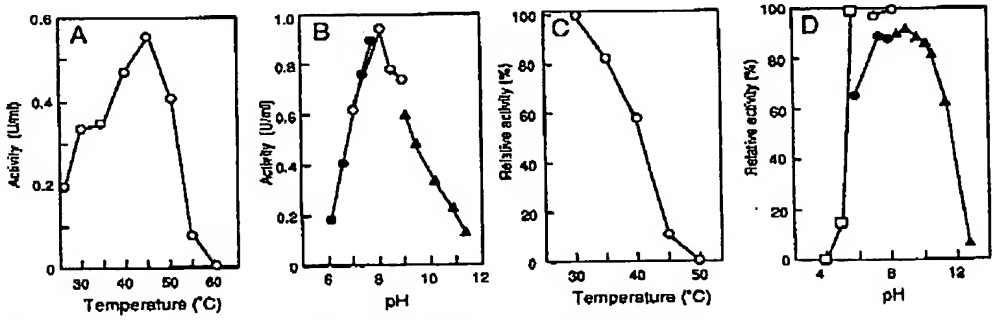

Fig. 3. Effects of Temperature and pH on Activity (A) (B) and Stability (C) (D) of FLOD.

(A) Enzyme activities were measured under the standard conditions at various temperatures.
(B) Enzyme activities were measured under the standard conditions in various buffers.
(C) The residual activities were measured after the purified enzyme was pre-incubated in 33 mM Tris-HCl buffer (pH 8.5) at various temperatures for 5 min.
(D) The residual activities were measured after the purified enzymes was pre-incubated in 33 mM various buffers at 30°C for 5 min.
Symbols in Figs. 3B and 3D: □, acetate buffer; ●, potassium phosphate buffer; ○, Tris-HCl buffer; ▲, glycine–NaOH buffer.

40°C and 8.0, respectively (Fig. 3A, B). The enzyme was inactivated gradually above 30°C and completely lost the activity at 50°C for 5 min (Fig. 3C). The enzyme was stable at a pH range from 6.0 to 10.0 (Fig. 3D).

Effects of metal ions and reagents

The enzyme activity was completely inhibited by Cu^{2+}, Zn^{2+}, Ag^{+}, and Hg^{2+} and partially by several other metal ions (Table IV). The enzyme was strongly inhibited by *p*-chloromercuribenzoate, hydrazine, and phenylhydradine (Table V). These findings suggest that sulfhydryl and carbonyl groups exist in the catalytic site of the enzyme and are important in the enzyme reaction.

Prosthetic group

The adsorption spectrum of the purified enzyme that had two absorption maxima at 373 and 454 nm (Fig. 4) is a typical one for flavoprotein. The absorption maximum at 454 nm disappeared when the enzyme was incubated with ε-FL. The AMP moiety of the chromophore was identified as follows: the purified enzyme was digested with Pronase, then AMP was liberated by phosphodiesterase treatment. As a result, 6.26 nmol AMP was found in 6.29 nmol FLOD indicating that one mole of FLOD had one mole of FAD as a prosthetic group. This FAD was not liberated from the enzyme with trichloroacetic acid precipitation and yellow fluorescence migrated along with the enzyme protein on SDS–PAGE. These findings suggest that the FAD can bind covalently to the protein.

Table IV. Effects of Metal Salts on FLOD Activity

Material (1 mM)	Relative activity (%)	Material (1 mM)	Relative activity (%)
None	100	$FeSO_4$	97
LiCl	100	$CoSO_4$	42
KCl	104	$CuCl_2$	0
NaCl	107	$ZnSO_4$	0
RbCl	103	$AgNO_3$	0
CsCl	102	$BaCl_2$	60
$MgCl_2$	75	$HgCl_2$	0
$CaCl_2$	77	$FeCl_3$	67
$MnCl_2$	154		

The enzyme was incubated for 5 min with a metal salt.

Table V. Effects of Various Reagents on the FLOD Activity

Reagent (1 mM)	Relative activity (%)	Reagent (1 mM)	Relative activity (%)
None	100	Semicarbazide	103
PCMB[a]	0	Phenylhydrazine	2.6
DTNB[b]	95	Hydrazine	13
Iodoacetate	102	Hydoxylamine	21
NaN_3	101	Clorgyline	132
α,α'-Dipyridyl	106	Deprenyl	102
o-Phenanthroline	103	Aminoguanidine	66

The enzyme was incubated for 5 min with a reagent.
[a] *p*-Chloromercuribenzoic acid.
[b] 5,5'-Dithiobis(2-nitrobenzoic acid).

Discussion

Since glycation of protein occurs mainly in the ε-amino group of the lysine residue, ε-ZL is a suitable model compound of glycated residues in proteins. We found one of the fungi imperfecti, *F. oxysporum* S-1F4, that produced FLOD by the use of ε-ZL as a sole source of nitrogen through the enrichment cultures.

Fructosyl amino acid (amine) oxidase had been purified from *Corynebacterium* sp.[18] and *Aspergillus* sp.[19] Table VI shows the properties of enzymes. The two enzymes were dimers with identical 44 kDa and 43 kDa subunits, respectively, while FLOD from *F. oxysporum* S-1F4 was found to be a monomer with a molecular mass of 50 kDa. *Corynebacterium* and *Aspergillus* enzymes were flavoproteins with non-covalently bound FAD, and FLOD from *F.*

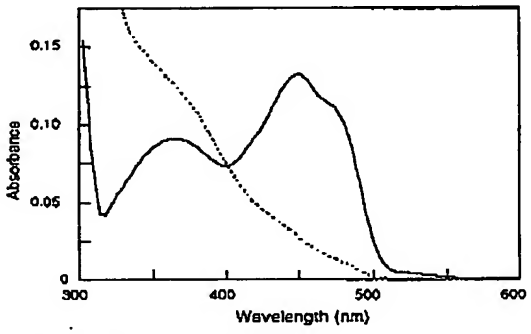

Fig. 4. Absorption Spectra of FLOD.
Solid line: Purified FLOD (0.20 mg/ml) in NY buffer; broken line: Purified FLOD (0.20 mg/ml) pre-incubated with 0.2 mM ε-FL in NY buffer at 30°C for 3 h.

Table VI. Properties of the Amadori Compound-oxidizing Enzymes from Various Microorganisms

	F. oxysporum S-1F4 (FLOD)	*Corynebacterium* sp.[18] (Fructosyl-amino acid oxidase)	*Aspergillus* sp.[19] (Fructosylamine oxidase)
Molecular Gel filtration	45,000	88,000	83,000
weight SDS-PAGE	50,000	44,000	43,000
Coenzyme	Covalently bound FAD	Non-covalently bound FAD	Non-covalently bound FAD
Specific Fructosyl lysine	48.9[a]	N.D.[b]	11.28[a]
activity Fructosyl valine (U/mg)	<0.01	7.09	59.8
Michaelis constant	0.22 mM (for ε-FL)	0.74 mM (for fructosyl glycine)	2.2 mM (for fructosyl glycine)
Optimum pH	8.0	8.3	7.7
Inactivation by SH-reagent	Yes	No	Yes

[a] Specific activity against ε-FL.
[b] Specific activity against N^{ε}-D-fructosyl-N^{α}-formyl-lysine.

oxysporum S-1F4 was also a flavoprotein but with covalently bound FAD. FLOD showed low activity against fructosyl valine, while this compound was a good substrate with the other two enzymes. FLOD from *F. oxyporum* S-1F4 had the same degree of activity against ε-FL and α-FL, but the *Km* was lower for ε-FL than for α-FL. Therefore, FLOD seems to recognize glycation in the ε-amino group. Recently, Watanabe *et al.* found an enzyme that catalyzes the deglycation of fructosyl-lysine[20] but the detailed properties of the enzyme have not been described. Thus, FLOD differs in the described properties from the other previously reported enzymes.

We also showed that the FLOD could act against fructosyl poly L-lysine and not against intact glycated protein. However, our preliminary experiments showed that protease treatment allowed FLOD to act against glycated proteins (data not shown). These findings suggest the possibility of the application of FLOD to the measurement of glycated proteins such as glycated albumin in the serum.

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FULL TEXT OF CASES (USPQ2D)
All Other Cases

Continental Can Co. USA Inc. v. Monsanto Co. (CA FC) 20 USPQ2d 1746 Continental Can Co. USA Inc. v. Monsanto Co.

U.S. Court of Appeals Federal Circuit
20 USPQ2d 1746

Decided November 13, 1991
No. 90-1328

Headnotes

JUDICIAL PRACTICE AND PROCEDURE

1. Procedure - Summary judgment - Patents (§ 410.3303)

Summary judgment is as available in patent cases as in other areas of litigation and can facilitate disposition of legally meritless suits, but improvident grant of summary judgment can prolong litigation and increase its burdens, especially in patent disputes in which patent property is wasting asset.

PATENTS

2. Patentability/Validity - Anticipation - In general (§ 115.0701)

Anticipation under 35 USC 102 cannot be found if more than one reference is required to establish unpatentability of claimed invention; rather, validity in such case is determined pursuant to 35 USC 103.

3. Patentability/Validity - Anticipation - Prior art (§ 115.0703)

Patent construction - Claims - Defining terms (§ 125.1305)

Federal district court erred by ruling, on summary judgment, that claims for patented bottle were anticipated by prior art, since court erred in its construction of claim term "hollow," and since disputed issue of fact exists as to whether injection blow molding process necessarily produced "hollow" ribs in prior art base structure, as term "hollow" is used in patent.

4. Patentability/Validity - Anticipation - Prior sale - In general (§ 115.0707.01)

"On sale" bar of 35 USC 102(b) does not arise simply because intended customer was participating in development and testing, but rather all circumstances concerning relationship between patentee and customer must be considered in light of public policy underlying Section 102(b); thus, federal district court erred in determining that bottle was "on sale," in view of evidence showing that bottle was part of terminated development project that never bore commercial fruit and was cloaked in confidentiality.

5. Patentability/Validity - Obviousness - Combining references (§ 115.0905)

Federal district court erred by ruling, on summary judgment, that claimed bottom structure for plastic container was obvious, since, drawing all reasonable inferences in favor of patentee, it has not been established that person skilled in art would be motivated to select and combine features from each prior art source to make patented base.

6. Patentability/Validity - Obviousness - Secondary considerations generally (§ 115.0907)

Differences between patented invention and prior art which may appear technologically minor nonetheless can have practical impact, particularly in crowded field, and in such case objective indicia, such as commercial success, or filling existing need, illuminate technological and commercial environment of inventor, and aid in understanding state of art at time invention was made.

7. Patentability/Validity - Obviousness - Commercial success (§ 115.0908)

Patented invention need not be solely responsible for commercial success in order for this factor to be given appropriate weight.

Particular patents - General and mechanical - Plastic bottle

4,108,324, Krishnajumar, Roy, Pocock, Das, and Mahajan, ribbed beverage bottle structure for plastic container created by plastic hot-fill, summary judgment of invalidity vacated in part, reversed in part, and remanded.

Case History and Disposition:

Appeal from the U.S. District Court for the Southern District of Ohio, Spiegel, J.; 11 USPQ2d 1761 .

Patent infringement action brought by Continental Can Co. USA Inc. and Continental Pet Technologies Inc. against Monsanto Co., Hoover Universal Inc., and Johnson Controls Inc. From federal district court decision entering summary judgment in favor of defendants, plaintiffs appeal. Vacated in part, reversed in part, and remanded.

Attorneys:

Eugene F. Friedman, Chicago, Ill. (Edwin C. Thomas, III and David M. Novak, of Bell, Boyd & Lloyd, Chicago; Kurt L. Grossman, of Wood, Herron & Evans, Cincinnati, Ohio, with him on brief), for plaintiff-appellants.

Henry J. Renk, New York, N.Y. (Lawrence F. Scinto and Bruce C. Haas, of Fitzpatrick, Cella, Harper & Scinto, New York; Jacob K. Stein and Deborah DeLong, of Thompson, Hine & Flory, Cincinnati, Ohio; Lawrence L. Limpus, St. Louis, Mo., and Edward L. Levine, Milwaukee, Wis., with him on brief), for defendants-appellees.

Judge:

Before Newman, Archer, and Rader, circuit judges.

Opinion Text

Opinion By:

Newman, J.

Continental Can Company USA and Continental PET Technologies (collectively "Continental") appeal the partial summary judgment of the United States District Court for the Southern District of Ohio, holding that United States Patent No. 4,108,324 (the Conobase or '324 patent) is invalid. 1 Final judgment was entered on this issue, for the purpose of appeal.

Summary Judgment

An issue may be decided on motion for summary judgment when there is no genuine issue of material fact, and the movant is entitled to judgment as a matter of law. Fed. R. Civ. P. 56(c); *Anderson v. Liberty Lobby, Inc.,* 477 U.S. 242 (1986); *Celotex Corp. v. Catrett,* 477 U.S. 317, 325-26 (1986); *Scripps Clinic & Research Foundation v. Genentech, Inc.,* 927 F.2d 1565, 1571, 18 USPQ2d 1001, 1005 (Fed. Cir. 1991). The movant's burden is to show that no fact material to the issue is in dispute, that even if all material factual inferences are drawn in favor of the non-movant the movant is entitled to judgment as a matter of law. *Id.* Summary judgment is as available in patent cases as in other areas of litigation. *Chore-Time Equipment, Inc. v. Cumberland Corp.,* 713 F.2d 774, 778-79, 218 USPQ 673, 675 (Fed. Cir. 1983)

The purpose of the summary process is to avoid a clearly unnecessary trial, *Matsushita Elec. Industrial Co. v. Zenith*

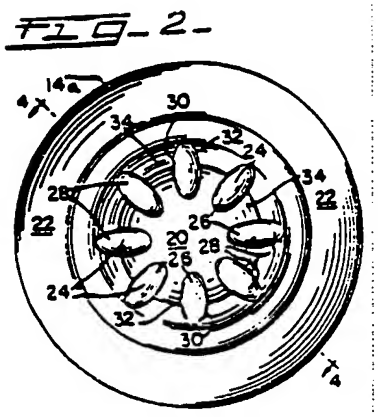
Radio Corp., 475 U.S. 574, 587 (1986); it is not designed to substitute lawyers' advocacy for evidence, or affidavits for examination before the fact-finder, when there is a genuine issue for trial. As stated in *Adickes v. S.H. Kress & Co.*, 398 U.S. 144, 176 (1970) (Black, J., concurring), "[t]he right to confront, cross-examine and impeach adverse witnesses is one of the most fundamental rights sought to be preserved by the Seventh Amendment". *See also Poller v. Columbia Broadcasting System, Inc.*, 368 U.S. 464, 473 (1962).

[1] While facilitating the disposition of legally meritless suits, when summary judgment is improvidently granted the effect is to prolong litigation and increase its burdens. This is of particular concern in patent disputes, where the patent property is a wasting asset, and justice is ill served by delay in final resolution. In the case at bar, although some issues could be resolved on the law and undisputed facts, other issues require trial.

The Patented Invention

The '324 patent, entitled "Ribbed Bottom Structure for Plastic Container", inventors Suppayan M. Krishnakumar, Siegfried S. Roy, John F. E. Pocock, Salil K. Das, and Gautam K. Mahajan, is directed to a plastic bottle whose bottom structure has sufficient flexibility to impart improved impact resistance, combined with sufficient rigidity to resist deformation under internal pressure. The patented bottle is said to provide a superior combination of these properties. The bottom structure is illustrated as follows:

Fig. 2



Claim 1 is the broadest claim of the '324 patent:

1. A container having a sidewall and a bottom structure closing the container at an end portion of the sidewall, the outer surface of the bottom structure comprising a central concavity,
a convex heel surrounding the concavity and merging therewith and with the sidewall end portion, the lowermost points of the heel lying in a common plane,
and a plurality of ribs interrupting the outer surface of the concavity and distributed in a symmetrical array, each rib extending longitudinally in the direction of the heel and downwardly from an inner portion of the concavity, whereby the outer end portion of each rib is lower than the inner end portion thereof,
characterized by the feature that the ribs are hollow.

Claims 2 through 5 include additional limitations, described as contributing to the structure's rigidity, flexibility, or both. Claim 2 specifies the ratios of thickness of the walls of the bottom structure to the thickness of the sidewall end portions. Claims 3 specifies that the margins of each rib merge smoothly with adjacent portions of the bottom structure. Claim 4 specifies that each rib is convex relative to the bottom structure. Claim 5 specifies that each rib is of fusiform (a gently tapered shape at the ends) configuration. Each claim carries an independent presumption of

validity, 35 U.S.C. § 282, and stands or falls independent of the other claims. *Altoona Publix Theatres, Inc. v. American Tri-Ergon Corp.*, 294 U.S. 477, 487 [24 USPQ 308] (1935).
Continental brought suit for patent infringement against Monsanto Company and Monsanto's successor in this business, Hoover Universal, Inc. and Hoover's parent company, Johnson Controls (collectively "Monsanto"). Monsanto moved for partial summary judgment based on issues of validity under 35 U.S.C. §§ 102 and 103.

I

35 U.S.C. § 102(a)

The statutory requirement that a patented invention be "new" is tested in accordance with 35 U.S.C. § 102(a), which provides that:
§ 102. A person shall be entitled to a patent unless-
(a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for patent....
The district court found that all the claims of the '324 patent were anticipated by U.S. Patent No. 3,468,443 (the Marcus patent). We conclude that the district court erred in claim interpretation, and also found disputed facts adversely to the nonmovant, thus inappropriately deciding the issue summarily.
[2] Anticipation under § 102(a) requires that the identical invention that is claimed was previously known to others and thus is not new. *Scripps Clinic,* 927 F.2d at 1576, 18 USPQ2d at 1010; *Titanium Metals Corp. of Am. v. Banner,* 778 F.2d 775, 780, 227 USPQ 773, 777-78 (Fed. Cir. 1985); *Lindemann Maschinenfabrik GmbH v. American Hoist and Derrick Co.,* 730 F.2d 1452, 1458, 221 USPQ 481, 485 (Fed. Cir. 1984). When more than one reference is required to establish unpatentability of the claimed invention anticipation under § 102 can not be found, and validity is determined in terms of § 103.
It was Monsanto's burden to show that every element of the several claims of the '324 patent was identically described in the asserted anticipating reference, the Marcus patent. The district court focused on the term "characterized by the feature that the ribs are hollow", which limits all of the '324 patent claims. Continental argues that the district court incorrectly construed this term, as a matter of law, and that the Marcus patent shows ribs that are not hollow, as that term is used in the '324 patent. Continental also points to other differences between the '324 claims and the description in the Marcus patent.
The Marcus patent rib structure is illustrated in Figure 5 and in cross-section in Figure 6:

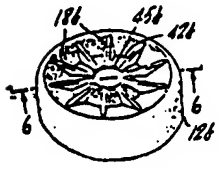


FIG. 5

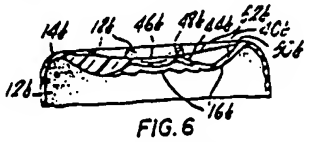


FIG. 6

The Marcus patent does not state that its ribs are "hollow", or use a similar term. Continental's witnesses testified by deposition that the Marcus patent shows solid, not hollow, ribs. A witness (Adomaitis) had stated in an internal memorandum written at Continental in 1969, well before this litigation arose, that "the ribs of their [Marcus'] web can be made of solid beams only." Another witness, '324 co-inventor Pocock, testified that:

It seems evident to me that he [Marcus] was trying to produce some kind of container integrity by the production of essentially solid ribs on the bottom of the bottle. It seems to go to great length here to illustrate them as such. Krishnakumar, another co-inventor, testified that it "is very obvious the ribs are shown solid", and that Figures 5 and 6 as well as Figures 7 through 12 of the Marcus patent all show solid ribs. However, Marcus, testifying for Monsanto, testified that his ribs were hollow, and that conventional blow molding would inherently produce hollow ribs.
The district court defined "hollow" as meaning that "the inside contour of the ribs generally follows the outside contour thereof", a definition on which the parties agreed. *Continental,* 11 USPQ2d at 1764. See the court's opinion, 11 USPQ2d at 1764-68, for various sketches made by the witnesses. Continental states that the district court erred in construing "hollow", and that the phrase "characterized by the feature that the ribs are hollow" must be construed in terms of the patent in which it appears. *See, e.g., Tandon Corp. v. United States Int'l Trade Comm'n,* 831 F.2d 1017, 1021, 4 USPQ2d 1283, 1286 (Fed. Cir. 1987). The '324 patent explicitly distinguished the Marcus teachings, stating that the '324 ribs are, unlike Marcus, not filled with plastic. The '324 specification uses the term "hollow", as do the prosecution history and the claims, for this purpose. The '324 patent's usage of "hollow" is illustrated in rib cross-section in Figure 5A:

Fig. 5A

The Marcus patent's rib structure thus was explicitly differentiated by the term "hollow" as used in the '324 specification, drawings, and prosecution history. Since the claim term must be construed as used by the patentee, the district court erred in its construction of the '324 claim term "hollow". On correct claim construction, the factual question of anticipation must be decided.
Monsanto's argument is that hollow ribs were inherently produced by Marcus. Monsanto thus argues that anticipation lies because the Marcus patent's ribs are "inherently" hollow, regardless of how they are shown in the Marcus patent. Monsanto argues that because the Marcus ribs are formed by injection blow molding, which is the same process described for the Conobase '324 ribs, hollow ribs are inherently disclosed in the Marcus patent.
To serve as an anticipation when the reference is silent about the asserted inherent characteristic, such gap in the reference may be filled with recourse to extrinsic evidence. Such evidence must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. *In re Oelrich,* 666 F.2d 578, 581, 212 USPQ 323, 326 (CCPA 1981) (quoting *Hansgirg v. Kemmer,* 102 F.2d 212, 214, 40 USPQ 665, 667 (CCPA 1939)) provides:
Inherency, however may not be established by probabilities or possibilities. The mere fact that a certain thing *may* result from a given set of circumstances is not sufficient. [Citations omitted.] If, however, the disclosure is sufficient to show that the natural result flowing from the operation as taught would result in the performance of the questioned function, it seems to be well settled that the disclosure should be regarded as sufficient.
This modest flexibility in the rule that "anticipation" requires that every element of the claims appear in a single reference accommodates situations where the common knowledge of technologists is not recorded in the reference; that is, where technological

facts are known to those in the field of the invention, albeit not known to judges. It is not, however, a substitute for determination of patentability in terms of § 103.

[3] Continental does not dispute the applicability of the injection blow molding process. However, Continental disputes the material of fact of whether this process necessarily produced "hollow" ribs in the Marcus base structure, as the term "hollow" is used in the '324 patent. Resolution of this disputed fact adversely to Continental was improper on summary judgment. The grant of summary judgment of anticipation under § 102(a) is vacated. The issue requires trial.

II

35 U.S.C. § 102(b)

The district court also held that the Marcus bottle was on sale, 35 U.S.C. § 102(b). Section 102(b) bars entitlement to a patent when:

(b) the invention was ... in public use or on sale in this country, more than one year prior to the date of the application for patent in the United States....

The Marcus bottle was developed some ten years before the filing date of the '324 patent, during a project wherein Marcus' employer, Admiral Plastics or APL Corporation, entered into agreements with the Coca-Cola Company for the development of a suitable plastic bottle. The agreements provided that Admiral Plastics would make and Coca-Cola would test the bottles, and that if a satisfactory bottle was developed it would be manufactured by Admiral and purchased by Coca-Cola. Minimum commercial quantities and maximum commercial prices were stated in an agreement, and costs were a matter of discussion. Admiral produced a variety of bottle shapes, including the Marcus bottle. The project was terminated after about two years, because the "mechanical performance" requirements were not met as Coca-Cola wrote at the time.

[4] The district court reasoned that this project "called for the eventual marketing of the Marcus bottles once all technical difficulties were resolved", *Continental,* 11 USPQ2d at 1766, and on this basis held that the Marcus bottles were on sale. This holding was in error, for the "on sale" bar of § 102(b) does not arise simply because the intended customer was participating in development and testing. *See Great Northern Corp. v. Davis Core & Pad Co.,* 782 F.2d 159, 164-65, 228 USPQ 356, 358 (Fed. Cir. 1986). In *Baker Oil Tools, Inc. v. Geo Vann, Inc.,* 828 F.2d 1558, 1563-65, 4 USPQ2D 1210, 1213-15 (Fed. Cir. 1987), this court summarized various factors pertinent to the "on sale" bar when there is an issue concerning the relationship between the patentee and the customer: for example, whether there was a need for testing by other than the patentee; the amount of control exercised; the stage of development of the invention; whether payments were made and the basis thereof; whether confidentiality was required; and whether technological changes were made. All of the circumstances attending the relationship must be considered in light of the public policy underlying § 102(b). *UMC Electronics Co. v. United States,* 816 F.2d 647, 656, 2 USPQ2d 1465, 1471-72 (Fed. Cir. 1987), *cert. denied,* 484 U.S. 1025 (1988).

The district court acknowledged that all technical difficulties were not resolved and that no sales were ever made. Although Admiral Plastics' hope was surely commercial sales, and the record shows that prices and quantities were discussed, this does not of itself place the subject matter "on sale" in the sense of § 102(b). The Marcus bottle was part of a terminated development project that never bore commercial fruit and was cloaked in confidentiality. While the line is not always bright between development and being on sale, see generally *UMC Electronics, supra,* in this case the line was not crossed. The "on sale" bar is measured by "the time the public came into possession of the invention", *id.* at 655, 2 USPQ2d at 1471 (quoting *In re Foster,* 343 F.2d 980, 987-88, 145 USPQ 166, 173 (CCPA 1965), *cert. denied,* 383 U.S. 966 [149 USPQ 906] (1966) ("What starts the period running is clearly the availability of the invention *to the public* through the categories of disclosure enumerated in 102(b). ..." (emphasis in original))). We conclude that the district court erred in holding that the circumstances that here existed placed the Marcus bottles "on sale" in terms of § 102(b). We therefore reverse and direct that on remand judgment on this issue shall be entered in favor of Continental, as a matter of law.

III

35 U.S.C. § 103

Obviousness, 35 U.S.C. § 103, is reviewed as a legal conclusion based upon underlying facts of four general categories, *viz.* the scope and content of the prior art, the differences between the prior art and the claimed invention, the level of ordinary skill at the time the invention was made, and any objective considerations that may be present. *Gra*

ham v. John Deere Co., 383 U.S. 1, 17 [148 USPQ 459] (1966); *Interconnect Planning Corp. v. Feil,* 774 F.2d 1132, 1137-38, 227 USPQ 543, 547 (Fed. Cir. 1985).

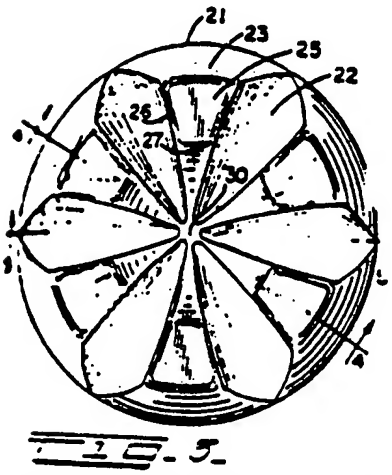
[5] The parties agreed that the scope and content of the prior art was adequately represented by four references: the Marcus patent discussed in Part I *ante,* a patent to Colombo (U.S. Patent No. 3,403,804), and two patents owned by Continental, U.S. Patent No. 3,598,270 (the Petaloid patent), and No, 3,935,955 (the Decaloid patent). They agreed on little else. In granting summary judgment of invalidity for obviousness, the district court found certain disputed material facts and misapplied certain precepts of law. We conclude that the issue was not amenable to summary resolution. Although it is not entirely clear how the references were combined by the court, we shall review the references briefly, in order to explain our conclusion.

The Petaloid Patent

The district court referred to the deposition testimony of Siegfried Roy, one of the co-inventors of the '324 patent, that the Petaloid base, inverted, was similar to the Conobase. Continental points out that neither Roy nor any other deponent suggested that the Petaloid base could be or should be inverted, or that inversion would provide an improved base structure. In *In re Gordon,* 733 F.2d 900, 902, 221 USPQ 1125, 1127 (Fed. Cir. 1984) this court held that although a prior art device could have been turned upside down, that did not make the modification obvious unless the prior art fairly suggested the desirability of turning the device upside down.

Continental points out that the Petaloid description differs in several other ways from the '324 invention. In the '324 structure the outer end of each rib is lower than the inner end, whereas in the Petaloid structure the outer ends of the ribs are higher than the inner ends; that is, the ribs in the Petaloid base extend upward from the center to the sidewall. The Petaloid bottle is supported on feet extending between the ribs, such feet being the locations for stress concentrations. The following drawing is from the Petaloid patent:

Fig. 3



Continental states that the '324 Conobase is not only different, but avoids the stress concentrations of the Petaloid

device, thus enhancing impact resistance. Monsanto argues that Continental simply used the Petaloid hollow ribs in combination with the Marcus patent. This requires determination of whether there was something in the prior art as a whole to suggest the desirability, and thus the obviousness, of making the combination, in a way that would produce the '324 structure. *See, e.g., Uniroyal, Inc. v. Rudkin-Wiley Corp.*, 837 F.2d 1044, 1051, 5 USPQ2d 1434, 1438 (Fed. Cir.), *cert. denied,* 488 U.S. 825 (1988). Continental argues that it is not apparent, even with hindsight, how any combination of the Petaloid and Marcus patents or other references lead to the '324 base. The Petaloid patent shows concave ribs that extend all the way to the sidewall, while the Marcus ribs extend "from the heel" toward an annular central ring. The Petaloid base has wide, petal-like, open ribs, while Marcus shows narrow, beam-like ribs. The deposition testimony was in conflict as to the inferences drawn from the references.
On this disputed issue, drawing reasonable inferences in favor of the non-movant, it has not been established that one skilled in the art would be motivated to select and combine features from each source in order to make the '324 base. *Interconnect Planning,* 774 F.2d at 1143, 227 USPQ at 551 ("When prior art references require selective combination by the court to render obvious a subsequent invention, there must be some reason for the combination other than the hindsight gleaned from the invention itself").

The Decaloid Patent

The district court also referred to combination of the Decaloid base with the Marcus base. The Decaloid base has ten hollow ribs that extend to the sidewall, and ten feet between the ribs:

Page 1752

Fig. 2

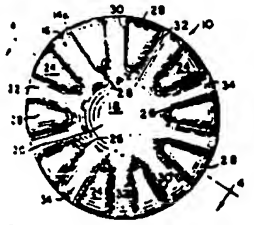


FIG-2-

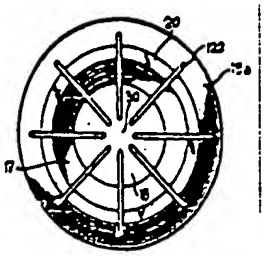
Monsanto does not explain, and we can not discern, how the combination with Marcus would have led a person of ordinary skill to the '324 base. The court's summary holding of obviousness based on these references, separately or in combination, can not be sustained.

The Colombo Patent

The Colombo base, like the Petaloid and Decaloid bases, has hollow ribs that extend to the sidewall, in a still different structure from that of Marcus and also from that of the '324 patent. Colombo describes his ribs as inverted U-shapes, concave, located on the outer surface of the central concavity:

Fig. 4

Fig. 4



Again, drawing reasonable factual inferences in favor of Continental, and in the absence of any suggestion or motivation in the prior art as a whole to make a selective combination of the Colombo and Marcus structures along with other changes needed to obtain the '324 structure, summary judgment of obviousness was inappropriate.
The district court found that there was no substantial difference between the '324 invention and the combined teachings of the prior art:
As obviousness can be established on the basis of the combined teachings of references, we think it is clear that simple enhancements of existing prior art, i.e., inverting the '270 petaloid base, do not constitute a substantial difference between the subject matter claimed in the '324 patent and that of the prior art. Thus, the facts of this case reveal no substantial difference between '324 and the prior art. *Continental,* 11 USPQ2d at 1769 (citation omitted).
However, as we have discussed, the criterion of § 103 is not whether the differences from the prior art are "simple enhancements", but whether it would have been obvious to make the claimed structure.

Objective Indicia

The district court concluded that the structure in suit is simply a variation on known themes. It is in such circumstance that the objective indicia - the so-called secondary considerations - are most useful to the decision-maker. The significance of a new structure is often better measured in the marketplace than in the courtroom.
[6] Thus when differences that may appear technologically minor nonetheless have a practical impact, particularly in a crowded field, the decision-maker must consider the obviousness of the new structure in this light. Such objective indicia as commercial success, or filling an existing need, illuminate the technological and commercial environment of the inventor, and aid in understanding the state of the art at the time the invention was made. *See In re Piasecki,* 745 F.2d 1468, 1475, 223 USPQ 785, 790 (Fed. Cir. 1984) (secondary considerations "often establish that an invention appearing to have been obvious in light of the prior art was not" (quoting *Stratoflex, Inc. v. Aeroquip Corp.,* 713 F.2d 1530, 1538-39, 218 USPQ 871, 879 (Fed. Cir. 1983))).
Continental licensed the '324 counterpart Japanese patent to a Japanese company, Yoshino, that we are told had been unable to develop a plastic bottle for hot-fill applications. A witness for Toyo Seikan, another Japanese licensee, testified that the Conobase "sustains itself in higher temperatures, and it does not cause buckling after you fill [the bottle]", as compared with previously available plastic bottles. Continental asserts that Monsanto had been unable to develop a satisfactory bottle for hot-fill applications, and had therefore obtained this technology from Yoshino.
[7] The district court acknowledged the commercial success of the Conobase, but stated that "we are not convinced that the conobase *alone* accounts for any of the success." 11 USPQ2d at 1770 (emphasis in original). The court suggested that the commercial success in Japan was due to the market strength of the Japanese licensees, and held that there is no nexus between the merits of the product and its commercial success. It is not necessary, however, that the patented invention be solely responsible for the commercial success, in order for this

factor to be given weight appropriate to the evidence, along with other pertinent factors. *See generally Demaco Corp. v. F. Von Langsdorff Licensing Ltd.,* 851 F.2d 1387, 1392-94, 7 USPQ2d 1222, 1226-28 (Fed. Cir.), *cert. denied,* 488 U.S. 956 (1988); *Rosemount, Inc. v. Beckman Instruments, Inc.,* 727 F.2d 1540, 1546, 221 USPQ 1, 7 (Fed. Cir. 1984). Monsanto also states that the Conobase is different from the '324 invention, so that even were the Conobase successful, this does not inure to the benefit of the '324 patent. It is apparent that the factual issues surrounding the objective indicia were disputed, and material.
In view of the material facts requiring resolution, the issue of obviousness was not properly decided on motion for summary judgment. We vacate the grant based on 35 U.S.C. § 103, and remand for trial of this issue and the other issues remaining in the case.

Costs

Costs in favor of Continental.
REVERSED IN PART, VACATED IN PART, and REMANDED

Footnotes

Footnote 1. *Continental Can Co. USA v. Monsanto Co.,* 11 USPQ2d 1761 (S.D. Ohio 1989), *reconsid. denied,* No. C-1-86-1213 (S.D. Ohio Nov. 9, 1989).

- End of Case -

